Evidence-Based Veterinary Medicine in Finfish Aquaculture in Newfoundland and Labrador

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Atlantic Veterinary College

University of Prince Edward Island

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Charlottetown, PE

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Examiners

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Date: June 26, 2012

Abstract

The Newfoundland and Labrador (NL) aquaculture industry is a major contributor of salmon products that help meet protein needs for the world's growing population. The industry in NL continues to grow and evolve and now includes emerging species in its production such as Atlantic cod. In a growing industry, with additional species being cultured, there are many opportunities to enhance evidence-based veterinary medicine (EBVM). The multifaceted process of EBVM includes critically evaluating published literature related to a particular question. Randomized controlled trials (RCT) are considered the best source of information with respect to interventions, but these trials are challenging to implement in an aquaculture setting. The aquaculture industry and their veterinarians need access to quality RCT in order to make sound, scientifically based health decisions. Therefore, it is the responsibility of the aquaculture industry and their veterinarians to assist in building this knowledge base to further advance the industry. The Newfoundland and Labrador Department of Fisheries and Aquaculture, together with the Centre for Aquatic Health Sciences, have worked with the NL industry and their veterinarians to answer questions while contributing to the process of EBVM.

The specific objectives of this research program evolved over time, but were all generally focused on the need for information in support of EBVM. The research focused on two objectives: evidence in support of trial execution and evidence from trials. The Passive Integrated Transponder (PIT) tagging study in Atlantic cod was developed to determine tag placement and evaluate adverse effects with such tag placement. This study was consistent with the first objective. The second objective was addressed by developing three clinical trials relevant to the NL industry at the time. These clinical trials were in response to questions around choosing treatment modalities for *Eubothrium crassum*, the option to use a nutraceutical during the smoltification stage of salmonid production and the use of a salmonid dip vaccine in Atlantic cod.

The tagging trial showed that there was no negative effect on survival and growth in Atlantic cod in the short term, thereby providing evidence to support the use of PIT tags in future studies.

The clinical trials showed (1) that treatment modalities adapted from terrestrial models do not provide predictable results when information is simply transferred, (2) that nutraceuticals need to be critically evaluated with respect to their label claims, and (3) that vaccination of Atlantic cod may provide protection against pathogens not included in the vaccine due to non-specific immunity.

The studies included in this thesis have contributed to the knowledge base used to inform aquaculture veterinarians who utilize EBVM. The results also highlighted techniques to obtain this information in an aquaculture setting.

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Mike and Thomas

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List of Abbreviations

ATPase	Adenosine Triphosphate
BA	Blood Agar
BMA	Bay Management Area
CHSE 214	Chinook Salmon Embryo 214
CI	Confidence Interval
EBM	Evidence-Based Medicine
EBVM	Evidence-Based Veterinary Medicine
FAO	Food and Agriculture Organization
GH	Growth Hormone
H&E	Hematoxylin and Eosin
HOBr	Hypobromous Acid
HPLC	High Performance Liquid Chromatography
HR	Hazard Ratio
NL	Newfoundland and Labrador
NL DFA	Newfoundland and Labrador Department of Fisheries and Aquaculture
NNV	Nervous Necrosis Virus
OIE	World Health Organization
PIT	Passive Integrated Transponders
RCT	Randomized Controlled Trials
RDVS-AVC	Regional Diagnostic Virology Services – Atlantic Veterinary College
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
Se	Sensitivity
SHK	Salmon Head Kidney
SKDM	Selective Kidney Disease Medium
Sp	Specificity
SSN-1	Striped Snakehead cell line
TMS	Tricaine Methane Sulfonate
TSA	Tryptic Soy Agar
VER	Viral Encephalopathy and Retinopathy
VI	Virus Isolation
VNN	Viral Nervous Necrosis

Chapter 1: Evidence-based veterinary medicine in aquatic animal practice

1.0 Introduction

The farming of Atlantic cod (*Gadus morhua*) and Atlantic salmon (*Salmo salar*) is an ongoing worldwide practice (Food and Agriculture Organization of the United Nations, 2009; Bricknell *et al.*, 2010). As areas of farming evolve, so does the care of these species. The tools available to the practicing veterinarian in aquaculture include evidence-based veterinary medicine (EBVM). Information gathered from randomized controlled trials (RCT) are essential to veterinarians who use EBVM as a tool in making decisions regarding treatment, mitigation and prognosis. The chapters in this thesis are divided into two categories: 1) evidence to support trial execution and 2) evidence of trials.

1.1 Introduction into aquaculture worldwide

The demands placed on capture fisheries by the world population have exceeded sustainability levels (Tidwell and Allan, 2001; Subasinghe, 2004). Demand for sources of protein for human consumption increases in step with the world's growing population, and fish protein has been identified as a key protein source. All forward projections indicate that meeting the health and protein needs of the world human population will require an increase in aquatic food products. Humans on a per capita basis consume fish more than they consume any other type of meat or animal protein (Subasinghe, 2004). This is largely influenced by the affordability of fish, particularly in developing countries. Fish as a source of protein can improve diets through increased consumption of fatty acids (Omega-3), vitamins and minerals (calcium, phosphorus, iron, selenium and iodine).

Aquaculture has grown rapidly to meet the increasing demands for aquatic products. Aquaculture production creates employment opportunities in rural and coastal communities, but needs to be affordable in relation to income and other protein sources (Tidwell and Allan, 2001; Bostock *et al.,* 2010; Thompson and Subasinghe, 2010). Subasinghe (2004) states, "The challenge is to develop approaches to increase the contribution of aquaculture, which are realistic and achievable, within the context of current social, economic, environmental and political circumstances." Sustainable aquaculture must be achieved while meeting the world's protein needs.

Aquaculture, as defined by the OIE (World Health Organization), is a "means of farming aquatic animals with some sort of intervention in the rearing process to enhance production, such as regular stocking, feeding and protection from predators". Aquatic animals are defined as "all life stages (including eggs and gametes) of fish, molluscs, crustaceans and amphibians originating from aquaculture establishments or removed from the wild, for farming purposes, for release into the environment or for human consumption" (Office International des Epizooties, 2009). Worldwide aquaculture production was reported to be 52.5 million tonnes in 2008 and worth $98.5 billion (USD). Asia, the largest contributor, accounts for 89% of production, with China contributing 32.7 million tonnes in 2008 (Bostock *et al.,* 2010). The Food and Agriculture Organization (The Food and Agriculture Organization of the United Nations, 2009) predicts that worldwide aquaculture will continue to increase; although this is not uniform by regions, production in Europe and North America are predicted to increase by at least 1% per year.

Excluding aquatic plants, 310 aquatic species are farmed worldwide. The top producing freshwater species include carp (*Cyprinus carpio*), tilapia (*Tilapia* spp.) and pangasius catfish

(*Pangasianodon hypophthalmus*). Marine species include shrimp (*Penaeus vannamei, P. stylirostris, P. monodon*), oysters (*Crassostrea virginica*), scallops (*Argopecten* sp.*, Chlamys* sp.*, Patinopecten* sp.*, Pectinopectin* sp.) and mussels *(Mytilus* sp.*, Perna* sp.*).* Globally, the leading species of intensively farmed marine finfish is Atlantic salmon (*Salmo salar*), a species that also contributes the greatest to aquaculture production — by volume and value — in Canada (Bostock *et al.,* 2010).

1.2 Aquaculture in Canada

Aquaculture in Canada dates back to 1857 in Eastern Canada when Atlantic salmon (*Salmo salar*) and brook trout (*Salvelinus fontinalis*) eggs were incubated and hatched. Oyster (*Crassostrea virginica*) production began in Prince Edward Island in 1865 (Canada, 2010). By the 1970s, commercial aquaculture in Canada had begun. Between 1986 and 2008, Canada's aquaculture production increased from 10,488 to 144,684 tonnes, worth $736 million (Canada, 2010). Aquaculture production (all species) in Eastern Canada (Quebec, Nova Scotia, Newfoundland and Labrador, New Brunswick and Prince Edward Island) had reached 67,742 tonnes by 2008 and was worth $339 million (Canada, 2010).

1.3 Aquaculture in Newfoundland and Labrador

The first aquaculture experience in NL was with an Atlantic cod (*Gadus morhua*) hatchery that was started on April 30, 1889 and completed by July 18, 1889. The goals for the hatchery were to produce fry that would then be released into the wild for enhancement purposes. A total of 186 million fry were reported to be released from that hatchery in 1896 (Baker *et al.*, 1992). Today the main cultured species in the province of Newfoundland and Labrador (NL) are Atlantic salmon (*Salmo salar*), rainbow/steelhead trout (*Oncorhynchus mykiss*), Atlantic cod and blue

mussels (*Mytilus edulis*). Atlantic salmon is produced in the greatest volume and has been in production in Newfoundland and Labrador since the 1970s. The NL aquaculture industry has experienced significant increases in the last three years, especially in the salmonid sector. In 2011, the total industry production exceeded 15,000 tonnes and had a value of over $120 million. The majority of this aquaculture production and associated value is from the salmonid sector. This level of production is expected to increase as companies continue to invest in expanding the infrastructure and capacity primarily along the south coast of NL. More than 1,000 people, both directly and indirectly, are employed in aquaculture production or processing, and most of these jobs are in rural areas of the province (Newfoundland and Labrador Department of Fisheries and Aquaculture, 2012).

In July 1992, the federal government placed a moratorium on fishing for northern cod stocks (NAFO Divisions 3JKL) in NL and 30,000 people became unemployed (Brown *et al.*, 2003). Commercial cod aquaculture was just beginning in NL and the Newfoundland and Labrador Department of Fisheries and Aquaculture (NL DFA) used this opportunity to partner with industry and academia to investigate the economic viability of cod aquaculture in the province. At the same time, other areas with major aquaculture production (e.g., Norway, Iceland and Scotland) were either developing Atlantic cod aquaculture or moving towards commercialization (Brown *et al.*, 2003; Rosenlund and Skretting, 2006).

The early development of cod aquaculture faced many complex challenges, including live feed delivery to young cod, proper nutrition for broodstock and juveniles, depth and structural requirements of marine cages, feeding strategies, optimal temperatures for growth, optimal site locations and novel pathogens causing clinical disease. As the cod industry expanded globally,

opportunities to investigate pathogens of concern naturally presented themselves. The NL DFA is responsible for the promotion, development and regulation of aquaculture in Newfoundland and Labrador. The NL DFA provided resources for health related clinical studies, many of which became chapters in this thesis.

1.4 Atlantic salmon production methods

In Eastern Canada, Atlantic salmon start the lifecycle in a freshwater hatchery where eggs are sourced from land-based (primarily) broodstock. The fish are hatched and then grown in freshwater on-land (in tanks, ponds or freshwater cages) until they smoltify. Smoltification, or parr-smolt transformation, is a biological process that is driven by the endocrine system. This process allows anadromous fish to move from freshwater to saltwater (Smith, 1993; Björnsson *et al.*, 2011). During their life in the hatchery, salmon are fed dry feed bought as pellets from commercial feed mills. The fry are weaned onto this diet directly after they have utilized their yolk sac. Most hatcheries in Eastern Canada participate in a veterinary surveillance program to determine freedom from and aid in early detection of pathogens as well as establish a health profile for the facility. Mortality removal in the hatchery setting is performed by using a dip net to remove them from the tank.

Salmon hatchery biosecurity protocols are generally designed to address the greatest hazards for introduction and spread of infectious pathogens. This includes cleaning and disinfection of equipment, sequence in which the animals are handled (sick fish last, young fish first), visitors, personal gear, mortality removal and feeding practices. While in the hatchery the fish are prepared for seawater entry and — under the advice of a licensed veterinarian, with the knowledge of endemic pathogens at the marine cage site — a vaccine will be chosen. Once

salmon reach approximately 25 g, they are vaccinated against seawater pathogens; the fish are anesthetized and injected with the vaccine into the intraperitoneal space. Following the appropriate timing for an immune response, they are then ready to enter the marine cage sites and are referred to as smolt (Björnsson *et al.,* 2011). Once transferred to the marine environment, most Atlantic salmon are maintained in floating cages that allow the exchange of water through nets to provide oxygen and remove water containing excretory products (e.g., ammonia, carbon dioxide). According to Halwart *et al.* (2007), these cages have been shown to be the most cost-effective production systems for a range of farm sizes and environments. At the marine cage site the fish are fed commercial pelleted feed by hand, feed blowers or a feed barge. Divers are used to collect mortalities at least weekly by collecting any dead or weak (moribund) fish at the bottom of the cages.

1.5 Atlantic cod production methods

Atlantic cod, a marine finfish, start and finish their lifecycle in saltwater. They are raised in saltwater hatcheries and fed a live feed diet until they are weaned onto a commercial pelleted feed at around 45 days post hatch. The live feed is usually comprised of *Brachionus* sp. (rotifers) and *Artemia* sp. (brine shrimp). These live food organisms are costly to maintain, and the nutritional content of this prey is variable. Live feed production is essentially another aquaculture species, required to produce cod. There have been many attempts to replace this live feed with a formulated diet, but poor acceptance of the diet by larvae, decreased growth rates and increased mortality during formulated diet trials have prevented a successful replacement from being viable (Bengtson, 2003; Puvanendran *et al.,* 2006). The incoming water into the hatchery is usually obtained directly from the ocean or via a commercial mix (e.g.,

Instant Ocean®). Water treatment is a little more challenging in that saltwater contains bromide and, if ozone treated, it may result in hypobromous acid (HOBr), which can then be further oxidized to bromate, potentially resulting in toxic brominated organics (Werner and Hogne, 1983). In addition, the water being used must be screened and particulates removed.

Cod are typically raised in on-land facilities until they reach a pre-determined size (5-100 g) and are then transferred to the marine site with floating cages (Svåsand et al., 2004). Before they are transferred to the cage site, they are sometimes vaccinated using a dip vaccine for bacterial pathogens (*Vibrio* Sp.). This process involves holding a group of cod in a net immersed in a vaccine bath for a period of time (according to vaccine label). The vaccination typically occurs when the cod weigh 5-10 g. This vaccine is a salmonid vaccine and not specific to Atlantic cod. In some jurisdictions, intraperitoneal salmonid vaccines are used in Atlantic cod aquaculture. Although salmon and cod lifecycles have similarities, they also have many differences: for instance, yolk sac size — Atlantic cod require prey (live feed) during juvenile development, while Atlantic salmon can be weaned onto a commercial pelleted diet (Smith, 1993; Brown *et al.*, 2003; Puvanendran *et al.*, 2006); survival rate — Atlantic salmon have higher survival rates compared to Atlantic cod (Brown *et al.*, 2003); smoltification — Atlantic cod do not undergo smoltification as they spend their entire lifecycle in saltwater (Brown *et al*., 2003; Svåsand *et al.*, 2004; Puvanendran *et al.*, 2006); and immune competence — Atlantic salmon have a more evolutionarily advanced immune system with predictable antibody responses to vaccines and pathogens (Whyte, 2007).

The immune system of the Atlantic cod relies on the innate immune system. The innate immune system is not fully understood and there is much debate in the literature regarding the function

of natural antibodies (Magnadóttir *et al.*, 2001; Lange *et al.,* 2005). Atlantic salmon mount a specific antibody response to a pathogen or vaccination. When applied to Atlantic cod, it is thought that vaccines only affect the non-specific immune system (Whyte, 2007).

Atlantic cod aquaculture has been affected by low prices, variable quality, slow growth and increased early sexual maturation (precocious) when compared to Atlantic salmon (Svåsand *et al.*, 2004). Researchers, producers and veterinarians worldwide have attempted several management options to address early maturation (e.g., photoperiod manipulation). Early maturation results in decreased growth, decreased fillet size and quality and increased growing time. In farmed cod this usually occurs two years after hatching, but males have been reported as early as one year, most likely related to good nutrition and favourable growth in an aquaculture setting (Svåsand *et al.*, 2004). This may have a negative effect on cod aquaculture worldwide. More research is needed in this area for cod aquaculture to be successful.

1.6 Evidence-based veterinary medicine

Aquaculture veterinarians play a crucial role in aquaculture production in both established and new emerging species. Evidence-based veterinary medicine (EBVM) is just one of the tools that aquaculture veterinarians will use in practice. EBVM is the process of integrating clinical expertise and the best available research evidence (Cohen, 2009). The Centre for Evidence-based veterinary medicine at the University of Nottingham states, “Evidence-based veterinary medicine is the use of best relevant evidence, in conjunction with clinical expertise, to make the best possible decision about a veterinary patient.” In addition, the circumstances of each patient, and the circumstances and values of the owner/care giver, must also be considered when making an evidence-based decision (Centre for Evidence-Based Veterinary Medicine,

2012). Sackett *et al.* (1996), defines evidence-based medicine (EBM) as "the conscientious, explicit and judicious use of current best evidence in making decisions about the care of individual patients". This process involves integrating individual clinical expertise (proficiency and judgment acquired through clinical experience/practice) with clinical evidence. Criticisms and fears have been raised in human medicine stating that the EBM process may result in decreasing clinical freedom. Veterinarians have a different view in that the term "evidence-based" promotes decisions based on scientifically sound evidence (Vandeweerd *et al.*, 2012).

One of the fundamental principles of EBVM requires that clinical activities must be based upon well-designed studies of disease events. There are five steps associated with a sound EBVM practice: 1) developing a clinical question; 2) searching for evidence to answer the question; 3) appraising the evidence gathered for validity, impact and applicability; 4) integrating the critical appraisal with clinical expertise; and 5) applying the answer to the patient and auditing of the outcome (Cohen, 2009; Tang and Griffiths, 2009; Vandeweerd *et al.*, 2012).

The first step, specifying an answerable clinical question, requires a specific but not too focused question. The main goal is to collect as much information relevant to that question without being overwhelmed with irrelevant data. In human medicine the acronyms PICO or PECOT have been utilized to help develop this question. The following list shows how the acronym is defined (Vandeweerd *et al.*, 2012):

- P - patient or the problem
- I or E - intervention or exposure
- C - control group
- O - outcome

- T- time frame in which the outcome is expected can sometimes be included

The second step, searching for the evidence, is a challenging task and should include the use of peer-reviewed journal articles as well as textbooks and internet publications. The third step, critical evaluation of the evidence, is a crucial step in the process and can be challenging when information is scarce.

Randomized controlled trials provide strong clinical evidence for step 2 of the EBVM process (Vandeweerd *et al.*, 2012). RCT are epidemiological studies where the investigator controls treatment and control groups through a formal randomized allocation process. Allocation to treatment or control groups can be accomplished by simple random assignment whereby each patient has a known probability of being distributed into each study group. Another approach more common in aquaculture settings is systematic random assignment whereby every n^{th} patient is selected to be in the treatment group (Dohoo *et al.,* 2009). Either of these procedures will result in the individuals being evenly distributed across the treatment groups, thus minimizing bias.

The final component of evaluating the evidence is determining the validity of the study results, the clinical importance and the relevancy to the patient or case (Cohen, 2009). Medina and Pailaquilen (2010) described the tools for evaluating research as falling into four categories: integrated review, systematic review, meta-analysis and methods combining qualitative research (meta-summary, meta-synthesis, meta-studies). The final two steps, the act of applying the answer to the patient and auditing the outcome, should be considered concurrently (Cohen, 2009).

Aquaculture has limited literature to assist with EBVM compared to human or terrestrial veterinary medicine. Utilizing RCT while developing and applying mitigation strategies/treatments will generate more evidence and better inform the EBVM process. However, this information must be published to increase the volume of valid information in the field of aquaculture veterinary medicine and to assist others in EBVM. Often with emerging pathogens or culturing new aquaculture species the research and clinical expertise is limited. Clinical field trials can be employed to enhance EBVM. In making decisions based on evidence, a following schematic of ascending rigor has been adapted from Rosenthal (2004) and Vandeweerd *et al.* (2012) (Figure 1.1).

Vandeweerd *et al.* (2012) also provides a guide as to which study designs are more adequately designed to answer specific questions. For example, if the question involves a treatment, then an RCT will be best suited to answer this question. However, if the question involves a prognosis, then prospective cohort study is the better study design. Of course in some cases the information available is not rich enough to offer this kind of choice. Gill *et al.* (1996) performed a retrospective study in human medicine to determine the proportion of interventions in general practices that are based on evidence from clinical trials. The authors reviewed case notes from one suburban training practice and found that 81% of interventions were based on evidence as defined by EBM. Only 31% of interventions were based on randomized controlled trial evidence, while 51% were based on convincing non-experimental evidence.

The concepts of EBVM have been applied by veterinarians for many years, with a multitude of examples in mastitis research, small animal practice and horse lameness. Ruegg (2010) states that it is often difficult to obtain rigorous research-based evidence, but that utilizing EBVM

principles can help guide treatment decisions. Ruegg highlights that well-designed RCT are needed to make more informed treatment decisions in veterinary medicine. Vandeweerd *et al.* (2012) does point out that in veterinary literature there are many more observational studies (longitudinal/cohort and case-control studies) than RCT. Expense may play a role, and observational studies may be preferred because the exposures are not easy to control for ethical and practical reasons. A well-designed observational study may be more useful than a poorly constructed attempt at RCT due to these constraints.

In the case of aquatic veterinary medicine, many health management practices are based on extrapolation from accepted science in other species, growing conditions or pathogen-host interactions. For instance, this thesis tested treatment for *Eubothrium crassum* parasite because producers and veterinarians assume the following: the tapeworm compromises the growth and/or survival of Atlantic salmon; treatment that has been applied to terrestrial species for similar parasites will have a beneficial effect on parasite burdens; similar treatments used in salmon have not shown obvious negative outcomes; and drug residue depletion will follow courses similar to other compounds used in salmon. However, critical analysis of the evidence demonstrates that these assumptions have little supporting evidence. This is a case of science that *should* work, not of that which *does* work.

1.7 Randomized controlled trials

1.7.1 Definition of randomized controlled trials

The terms “randomized controlled trials” and “randomized clinical trials” are sometimes used interchangeably. Some authors restrict the definition of clinical trials to those that involve

therapeutic products or those carried out in clinical settings. Randomized controlled trials are sometimes referred to as clinical trials when an intervention has been applied. This intervention may include prophylactic, diagnostic and/or therapeutic agents/procedures (Brown, 2005; Dohoo *et al.,* 2009). Dohoo *et al.* (2009) define controlled trials as those experiments that are planned and designed to evaluate products or procedures in subjects outside the laboratory. The factor to be investigated is referred to as *the intervention* and the end result is *the outcome*. Woodward (2008) discusses intervention studies as studies in which the investigators assign the treatment and can allocate "treatment groups" to avoid bias. Woodward goes on to state that most intervention studies are actually pharmaceutical studies or clinical trials due to the practicality of applying a treatment/intervention to a group (particularly to human subjects). Essentially, then, intervention and clinical trials are both forms of RCT.

Field trials are also RCT, but the subjects are defined by the initial occurrence of an outcome of interest (Rothman *et al.,* 2008). For example, when testing a fish vaccine in the field, the primary outcome of interest is the prevention of clinical signs associated with the pathogen contained in the vaccine. Field trials often require larger sample sizes and cost more than laboratory or tank trials. Sometimes the only viable option to study the outcome of interest (e.g., the effect of a vaccine when animals are naturally exposed to the pathogen) is in the field (Rothman *et al.,* 2008). In this application, investigators must consider the fact that the animals may not be exposed to the pathogen of interest; therefore, other outcomes of interest should be recorded as well, such as growth and survival. For example, a vaccine trial may result in animals that are not naturally exposed to the pathogen, and so the effectiveness of the vaccine in the field cannot be evaluated. Instead, potential side effects or impact on growth and survival may be the outcome of interest in this particular case.

Randomized controlled trials covers the terms clinical, field and controlled trials, and all of these must follow the same "rules". The key design elements of RCT are as follows (Dohoo *et al.,* 2009):

a. The objectives — The objective of the study must be clear and succinct.
b. The study group — The study group must be comprised of a source population representative of the target population to which the trial is applied. For example, if an RCT are designed to assess the efficacy of a sea lice treatment at marine cage sites, then the fish and their sea lice infestation must be typical of a production site.
c. The unit of concern — The level at which the intervention is applied (i.e., individual animal, farm or country). Sea lice treatments can be applied at a bay level while antibiotics are often applied at the site level.
d. The eligibility criteria — This refers to the factors used to select the study subjects. A narrow set of standards may increase the statistical power but decrease the generalizability of the trial. A more inclusive set of standards may decrease the statistical power but increase the ability to extrapolate the trial results to a larger group or population. For example, subjects obtained through a referral hospital who had specific diseases ruled out and with a set list of clinical signs will be more specific than any animal admitted to the hospital with general symptoms.
e. The sample size — Appropriate sample sizes are required to ensure the validity of a trial. In addition, Type I and Type II errors must be carefully considered. Type I errors occur when the null hypothesis is rejected when it is in fact true, and Type II errors occur when the null hypothesis is not rejected ("accepted") but in fact the alternative hypothesis is true. For example, a type I error would be one in which there is stated difference in survival between vaccine groups when in fact there is not. A type II error

occurs when it is concluded that there is no difference in survival between vaccine groups, when in fact there is. Type I error will be decreased by increasing the confidence level of the test. As statistical power increases, type II error decreases. Power refers to a study's ability to detect an effect of a given magnitude. Power can be increased by increasing sample sizes. Attention to clusters or subgroups must be considered at this stage. If there are groups of animals (same province, same bay, same farm, same cage), then they will be similar. If all the samples are obtained from this similar group, then the results will not reflect the entire population, which is made up of several provinces with several bays and several farms with several cages.

f. The allocation of study subjects — To prevent bias, a formal randomization process is required to allocate each subject (or group of subjects) to the intervention. Attention to clustering and subgroups during this process, and subsequently during the analysis, is essential. For example, an aquaculture study may involve many fish within many cages within many sites within many bays within a province or within many provinces. To allocate the first half of the fish removed from a tank or a cage to one study group and the remainder to another would create bias. The first group may be smaller or may be ill. Therefore, this study group would actually reflect the fact that they were easier to catch than the larger and healthier fish.

g. The specification of intervention — The intervention must be clearly and succinctly defined. For example, specification would include vaccination with vaccine A or treatment with chemotherapeutant A at dose A for a set duration administered in a set prescribed manner.

h. The masking or blinding — This is the process by which blinding occurs at some level of the trial to reduce bias related to sampling allocation, measurement or follow-up bias.

Trials can be single-, double- or triple-blinded. Single-blinded trials refer to those in which the participant (or owner/caregiver) is unaware of the intervention being applied (more applicable to human studies). Double-blinded trials refer to trials in which the participant (or caregiver) and selected members of the study team are unaware of the intervention. Triple-blinded trials have the participant (or caregiver), study team and those analyzing the results unaware of the intervention being applied (Dohoo *et al.*, 2009).

i. The follow-up/compliance — All groups must be followed rigorously and equally. For example, all micro-chipped fish within a study cage must be accounted for whenever possible. Mortalities removed from a cage site are all scanned and physical characteristics recorded (weight, length, sex) as well as standard diagnostics performed. For example, if you have a group of fish in a marine cage site that is part of your study and the information collected on mortalities is not consistent throughout, then the study may be unable to offer insight into when important event occurred (e.g., disease outbreak). In addition, mortalities can be lost to predators or cannibalism. Therefore, the tag is not recorded as mortality but as a missing data point.

j. The measuring of the outcome — One or two clinically relevant outcomes and one to three secondary outcomes should be recorded. Measured outcomes can be continuous, categorical or time-to-event and may be at a single point in time or at multiple time points. Outcomes should be limited, preferably to one or two, so that the logistics of data collection does not compromise the validity of measurements.

k. The analysis of trial results — Analysis can be carried out on an intent-to-treat or per-protocol basis. The differences between these two are that the intent-to-treat method

includes all data, while the per-protocol refers to those subjects or groups that complied and completed the study.

l. Ethical considerations — In Canada, all studies must comply with the Canadian Council on Animal Care (CCAC), which oversees animal use (including fish) and addresses many topics such as husbandry, welfare and stopping rules. Habing and Kaneene (2011) recently published a report discussing stopping rules for randomized controlled trials including reasons to stop a trial.

Advantages of RCT include the fact that the 'cause' precedes the 'effect' and thus potentially distorting variables (confounders) that are unknown or unmeasured can be distributed evenly across the groups using random processes. This minimizes confounding, and treatments can be compared with sufficient statistical power to reasonably detect a difference of biological or practical importance. RCT have challenges similar to a cohort study: data may be collected prospectively and therefore require careful planning to include relevant outcomes; there are ethical problems with giving a treatment/intervention to a group (particularly if the researcher is aware of undesirable effects); and excluding subjects or groups (e.g., based on stage of production) cause difficulties in generalizing results (Rothman and Greenland, 1998; Woodward, 2008).

Challenges in applying RCT to aquaculture are related to the logistics of fish farming and prospective data collection. Cages are stocked with fish from the same year class and usually the same hatchery. This is necessary for both logistics and biosecurity reasons, but it will result in excluding groups from the study. For example, many jurisdictions will stock a Bay Management

Area (BMA) with one year class of fish, and, if a novel pathogen occurs in that BMA but not in others, then your studies will be limited to that year class in the field setting. Another consideration in aquaculture veterinary medicine is that novel pathogens and newly emerging aquaculture species make it difficult to predict parameters that should be recorded.

1.7.2 Study validity

The study validity is affected by bias. Bias can occur in three main ways: confounding, selection and information bias (Dohoo *et al.*, 2009). Confounding bias is created by confounding variables that can be known or unknown and must be associated with both the exposure and the outcome. Estimating associations between exposure and outcome will not be accurate if the confounder is not controlled or accounted for. Any measure association between the outcome and the exposure may become biased as a result. For example, sex and sexual maturation are both potential confounders in studies involving farmed fish because the risk of disease will often be different based on the sex and sexual maturation status of the fish. If we ignore sex and sexual maturation, the outcome may be associated with a factor more related to selecting a specific sex/maturation group (e.g., sexually mature males) than actual disease. Therefore, recording the number of females and males in a group or assigning the exposure/treatment groups equally among the sexes will help adjust for or control the confounder. Not all confounders are known or can be measured, and so a randomization is the best way to balance these factors evenly to both the exposed and non-exposed groups (Dohoo *et al.*, 2009).

Selection bias is related to the procedures used to obtain the study participants and factors that influence their participation in the study. For example, if individuals are lost to follow-up and they become missing data points, then this may result in selection bias. Information bias refers to incorrectly classified information, such as measurement error. The sensitivity and specificity of a diagnostic test plays a crucial part in this information bias. The better the sensitivity and specificity (closer to 1), the less likely that information bias will occur due to measurement error. (Rothman *et al.,* 2008; Dohoo *et al.,* 2009).

1.7.3 Quality of controlled trials in the literature in veterinary medicine

The "gold standard" for evidence to support efficacy under conditions of use, particularly when it involves interventions with a chemotherapeutant, is RCT. To assume that published or pharmaceutical trials meet all the required criteria for RCT would not be accurate; each article must be critically evaluated during the information-gathering component of the EBVM. The final outcome from the EBVM will be flawed if this step is skipped or skimmed over (Brown, 2005; Simoneit *et al.,* 2011). A study conducted by Simoneit *et al.* (2011) evaluated veterinary literature available for two on-line sources in 2009. They evaluated the studies using controlled trial criteria described previously and reported that only 17% of the published studies met the above-mentioned criteria. Although aquatic veterinary practice has never been similarly evaluated, the multitude of species and conditions of use suggests that RCT are fairly rare. Furthermore, the complexity of performing RCT at fish farms creates additional challenges. This thesis explores many of the aspects of field trials in NL aquaculture where new species (Atlantic cod) and different growing conditions for established species (Atlantic salmon) present challenges to EBVM.

To address deficiencies and inconsistencies in reporting and writing, a set of standards referred to as the CONSORT (Consolidated Standard of Reporting Trials) statement was first published in 1996, revised in 2001 (Moher *et al.*, 2001) and further revised and published by Schulz *et al.* (2010). O'Connor *et al.* (2010) published a modified CONSORT statement referred to as the REFLECT statement (reporting guidelines for randomized control trails in livestock and food safety). This modification was developed to address the issues encountered when applying the CONSORT statement to livestock and food safety trials.

1.8 Clinical investigation

Clinical investigation in the field of veterinary medicine using EBVM to make clinical decisions is the focus of this thesis. As commercial production in Atlantic salmon and other species (primarily Atlantic cod) increased, RCT were necessary to provide further evidence for sound policy and management decisions. These opportunities arose as health issues presented themselves within the expanding industry. Clinical diseases and their mitigation strategies relevant to the NL aquaculture industry are outlined in the following sections.

1.8.1 Evidence to support trial execution

1.8.1.1 Passive Integrated Transponder (PIT) tagging in Atlantic cod

To minimize animal use in RCT, increase statistical power, and to evaluate fish on an individual fish level, PIT tagging was used in this thesis. PIT tags are internal microchip tags that have a unique alphanumeric number, which is displayed on a scanner (when activated). Passive Integrated Transponder tags are used in many surveillance and research situations to identify

individuals and enable recording of precise information on growth, survival, migration patterns, behaviour, fine spatial movements, habitat preferences, pedigree, and many other applications (Gibbons and Andrews, 2004; Gheorghiu *et al.,* 2010). PIT tagging had been used in previous study designs in Atlantic salmon (Burnley *et al.,* 2010) but rarely in Atlantic cod. The first objective of my study was to determine PIT tag placement in cod given their relatively small abdominal space and large liver. The second objective was to determine if there was any difference in growth and survival if PIT tags were used in a population. By demonstrating to the producer that PIT tagging could be performed on Atlantic cod with minimal risk or impact on growth and survival over the short term, the use of this identification technique could be employed in future RCT in support of further EBVM in cod.

1.8.2 Evidence of trials

1.8.2.1 Nodavirus survival analysis

Nodavirus control usually involves early detection and subsequent depopulation. Although vaccination is not yet available, most producers will use salmon vaccines as a protection against general disease or pathogen challenges. This practice is not based on any valid evidence but on the assumption that it may help and is not likely to have negative consequences. However, Atlantic cod immunology is not as well understood as that of higher teleost species, and cod do not typically mount an immune response with antibodies after vaccination (Whyte, 2007). Although vaccination in cod may stimulate the immune system and therefore provide protection for a period of time against a wide range of pathogens, the response is non-specific and difficult to quantify using routine methods (e.g., antibody titres).

Since the decisions to vaccinate cod are based on extrapolations from other species, a project was established to examine evidence that cod going through a natural outbreak of nodavirus had differential survival if previously vaccinated. This information will further enhance the information available for EBVM decisions in cod health management.

1.8.2.2 SuperSmolt™ evaluation

Production management in food-producing animals involves identifying the most financially efficient management methods while minimizing the potential for negative health or welfare consequences. Drugs are regulated by Health Canada if they make claims to alter health, while nutraceuticals are considered natural, dietary, or environmental components even if they improve growth of animals.

Nutraceuticals (with varying definitions around the world) are usually classified as both a food and a drug. However, they are regulated as food in most cases, and thus the regulatory requirements of proving that the product is both safe and effective are less strict than if they were classified as drugs (Dzanis, 2008). There is no requirement that these products undergo standardized assessments of efficacy in support of their claims, safety, or withdrawal times (i.e.,rigorous trials). Information to assist decisions by producers and their veterinarians regarding justification of the cost and safety of these products is sporadic, and the standards are inconsistent.

SuperSmolt™, currently supplied by Europharma, is not considered a drug, and its use is promoted in the Atlantic salmon aquaculture industry as a means to enhance the osmoregulatory transition of Atlantic salmon smolt as they are transferred to seawater. Some of the claims of SuperSmolt ™ include earlier attainment of standard size, larger fish at the time of transfer, synchronized smolt transfer schedule, and increased survival with decreased risk of disease after seawater transfer. The SuperSmolt ™ process involves water treatment with naturally available minerals and specially formulated feed (Europharma, 2012). These claims are considered beneficial if true, but, without independent supporting evidence that critically evaluates the product, it is difficult to recognize the product's true value as a health management tool.

1.8.2.3 *Eubothrium crassum* treatment evaluation

Eubothrium crassum, a cestode found in the pyloric cecae and proximal intestine of wild and farmed Atlantic salmon, has a wide distribution in marine and freshwater environments (Kennedy, 1978). The adult cestode can lead to decreased feed conversion ratio, increased cost of production, and extended grow-out cycle, and can impact the overall health of the animal (Mitchell, 1993). Chronic infection with *Eubothrium crassum* on aquaculture farms appeared to result in a 10-20% reduction of growth in Atlantic salmon (Mitchell, 1993). Bristow and Berland (1991) reported that infection with *Eubothrium* sp. resulted in a 10% reduction in growth of infected groups of farmed salmon. There is some evidence that parasitism with *Eubothrium crassum* increases susceptibility to other pathogens (Bristow and Berland, 1991). For these obvious reasons, when a naturally occurring infection with *Eubothrium crassum* was discovered in farmed Atlantic salmon, control options were required. The two drugs used in aquaculture contained the active ingredients praziquantel or fenbendazole.

In Canada, treatments available to aquaculture veterinarians for this parasite are only available through extra-label drug use (Health Canada, 2012). Dose, treatment frequency, efficacy and withdrawal times are all the responsibility of the prescribing veterinarian. Anecdotal evidence supported the use of either drug, but not which dose response was most effective. The actual product used in the study was based on the availability of the product, and other aquaculture veterinarians based the dose on the most frequent recommendations. Fortunately, the particular group of salmon experiencing natural infections had been previously PIT tagged and therefore provided the opportunity to critically evaluate the effect of this drug compared to control (no) treatment.

The decision regarding treatment choice was based on available scientific literature and expert opinion. To support treatment decisions, an RCT was initiated to address the question "Does it work?" in order to avoid simply relying on assumptions that it should. Results from this trial will now inform the EBVM process for *Eubothrium crassum* control in NL.

1.9 Concluding remarks

Veterinarians in many fields of veterinary medicine often employ EBVM in an informal way. It is essential that well designed, peer-reviewed RCT are published to ensure that quality information is available to those making decisions on clinical interventions. In the practice of veterinary medicine applied to aquatic animal health, RCT and other sources of information on which to base management decisions are scarce. The research questions for this thesis were driven by the needs of the practicing aquaculture veterinarians in NL who utilized EBVM to make informed decisions.

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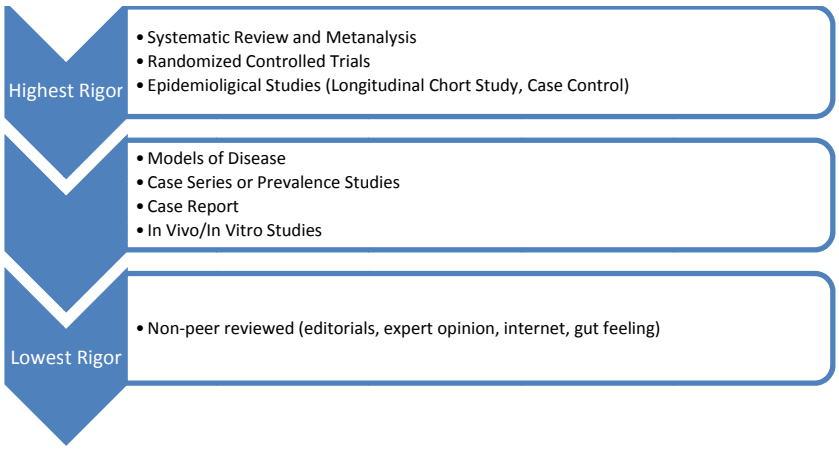
Figure 1.1: Schematic for decision-making in EBVM (adapted from Rosenthal, 2004 and Vandeweerd *et al.,* 2012).



Chapter 2: Passive Integrated Transponder (PIT) tag placement in Atlantic cod (*Gadus morhua*): a blinded randomized controlled trial to compare growth and survival over an 8-week period.

2.0 Introduction

Passive Integrated Transponder (PIT) tags are used in many surveillance and research situations to record precise information on growth, survival, migration patterns, behaviour, fine spatial movements, habitat preferences, pedigree as well as other applications (Gibbons and Andrews, 2004; Gheorghiu *et al.,* 2010). PIT tags have been used in aquatic and terrestrial animal applications for both farmed and wild populations (Parker and Rankin, 2003; Lee *et al.,* 2009, Sykes *et al.,* 2012). The tag is lightweight (~0.06 g), 2 mm in diameter and 12 mm in length (Gheorghiu *et al.,* 2010). It is composed of a biocompatible glass with an encapsulated copper antenna coil. The tag remains dormant until activated by an external reader using an electromagnetic field at a specific frequency. A signal is then generated, which is converted into a unique alphanumeric code displayed on an LCD screen or transferred directly to a computer. The tag is passive, does not require batteries or power and can only be activated by the reader (Mahapatra *et al*., 2001). Gibbons and Andrews (2004) summarized many uses of the tags and species that have benefited from their use. There are many internal tags available on the market, but this paper refers to the tag described above. To date, there have been no published articles on the use or placement of PIT tags in Atlantic cod (*Gadus morhua*).

PIT tags are internal markers, and are usually placed into the peritoneal cavity (intraperitoneal), the muscle (intramuscular) or just beneath the skin of the animal (subcutaneously) (Acolas *et al.,* 2007; Gheorghiu *et al.,* 2010). Gries and Letcher (2002) noted that there were two techniques

for tag placement in fish species: the first procedure utilizes a hypodermic needle, while the second uses a surgical implantation using a scalpel.

In a preliminary study, 40 Atlantic cod weighing 100 g were PIT tagged using both a surgical technique and placement using the AVID-supplied needle and syringe (O'Brien, 2005, unpublished). The fish were maintained in a tank for 4 weeks post-placement. For both groups, tag retention was 100%, and no mortality was noted immediately following the procedure. The AVID-supplied needle and syringe application resulted in rapid dulling of the needle when compared to the scalpel. The AVID needle and syringe application had to be replaced every 5^{th} fish; the scalpel had to be replaced every 10^{th} fish.

The surgical technique was chosen for this study. This was partially due to the dulling of the needle, but may also have been influenced by the researcher's experience with general surgery and therefore the comfort level with a scalpel. Alternatively, the needle and syringe could have been changed frequently, but this would have been more costly compared to the use of the scalpel. We speculated that any instrument that is dull or tears the skin would result in an increased potential for physical damage to the fish. Physical damage can potentially lead to secondary pathogen introductions, which could increase the risk of mortality. Although not attempted by the researchers, a sterile probe that can create a hole about the size of the PIT tag was also a possibility. The author strongly recommends that any individual PIT tagging for the first time use equipment they are comfortable with and that they seek training and practice before attempting this on any population of fish.

Several studies have shown that the tags do not affect growth or survival in other finfish such as Atlantic salmon (*Salmo salar*), Sea bass (*Sparus auratus)* (Gries and Letcher, 2002; Navarro *et al.,* 2006) and brown trout (*Salmo trutta*) (Ombredane *et al.,* 1998). Due to the economic importance of the marketable muscle tissues, PIT tags are often placed into the intraperitoneal space of these food species. Atlantic cod have a large liver and less abdominal space compared to salmonids. The tag would need to be placed in a manner that avoided penetrating internal organs and also in a way so that the liver could be avoided. This study was conducted to determine PIT tag placement and short-term effects on growth and survival in Atlantic cod. It was hypothesized that PIT tag placement into the intraperitoneal space of an Atlantic cod would not affect growth and survival post-tagging.

2.1 Materials and methods

2.1.1 Study population

A clinically healthy population of 600 juvenile Atlantic cod, 6-18 g in weight, maintained in a hatchery in Newfoundland and Labrador, was available for this study. All experimental fish were in good overall health prior to the start of the study. This was determined by a fish health evaluation on a subgroup (n=50) of the population prior to study commencement. General fish health was evaluated using histopathology, virology, bacteriology and parasitology. The size of fish examined in this study was based on previous experience by the investigators and current literature suggesting the smallest size feasible for initiation of tracking individual fish (Ombredane *et al.,* 1998). To control for the possible effects of handling and the impact of an incision, three groups were created with 200 fish in each one. The first group (PIT tag insertion

group) received an incision and a PIT tag. The second group (incision-only group) received an incision-only. The final group (control group) was handled, but received no incision or PIT tag.

2.1.2 Fish allocation

The fish were allocated into each treatment group using simple random assignment. A list of unique numbers was generated randomly using the computer-based statistical program STATA (version 10) software (StataCorp, STATA, College Station, TX). These numbers were assigned to each fish as they were removed from the source tank and then allocated to the study groups. The entire study population, from the first to the last fish entering the study, was evenly distributed between the three treatment groups.

2.1.3 PIT tagging methods

As per usual handling protocols at the hatchery, the Atlantic cod were starved for two days prior to tagging. The area was prepared by hanging tarps around the workspace prior to the tagging procedures. This would ensure that the researchers and those caring for the fish would not know which group was placed into which tank. In addition, individuals involved in placing the fish into the final three tanks were not also caring for the fish. The fish were anesthetized with tricaine methanesulfonate (TMS) at a concentration of 2.5 g/5 L of filtered seawater until they achieved dorsal recumbency. The fish were then placed in a holding container of filtered seawater. The fish were either held in the individual's hands for 5 seconds or an incision was made. A #12 scalpel blade with a guard made of plastic tubing (to prevent the incision from going too deep) was used to make an incision approximately 2 mm long on the ventral aspect of the fish on the left side of midline and 2 mm cranial to the anal pore (Figures 2.1 and 2.2). The

scalpel blade was sterilized in 70% isopropyl alcohol between each fish and replaced when dull (approximately every 10th fish). The individual making the incision was blinded to the remaining procedures and therefore did not know if the incision was actually made to place a PIT tag or if the fish was destined for the incision-only group.

The next stage in the process involved either placing a PIT tag into the fish or holding the fish for a further 5 seconds (the length of time to PIT tag a cod) to simulate the handling process used for implanting the tag. For PIT tag insertion, the tag was pushed into the abdomen with a finger and then gently palpated to confirm that the PIT tag was inserted fully (i.e.,not partially protruding from the abdominal wall). This palpation was not performed on the other two groups of fish. Sutures were not used to close the wound, as the insertion area was so small that the procedure of placing the sutures would be more traumatic to the fish than to leave the wound open. Weights and lengths were obtained for every fish and the PIT tag number recorded for those individuals with one placed. The tag was read with a portable handheld reader (AVID Power Tracker II, Calgary, Alberta, Canada) and recorded into a spreadsheet on a computer. A tank of aerated, filtered seawater was used as a recovery bath. Radiographs were obtained on a small subset of tagged fish (n=20) as soon after placement as possible to illustrate the typical PIT tag placement post-tagging.

2.1.4 Tank grow-out conditions

Once recovered, the fish were placed into one of three separate 6 L tanks, each holding one of the three treatment groups. Neither the investigators nor those caring for the animals were

aware of the fish treatment designations, as separate staff were responsible for removing and recording the mortalities.

2.1.5 Fish monitoring

Environmental conditions (such as oxygen, salinity and temperature), appetite and mortalities were monitored daily. The environmental parameters were all within normal limits. The mean oxygen, salinity and temperature were 100.39% (standard deviation: 8.93), 32 ppt (standard deviation: 0.024) and 13.57°C (standard deviation: 0.51), respectively. Weight, length, tank identification and PIT tag number (if applicable) was obtained for each fish mortality. Fish health assessments were conducted by performing a necropsy and obtaining appropriate tissue samples for virology, histopathology, bacteriology and parasitology on moribund fish. The fish were followed for 8 weeks, and weight, length, sex and treatment group were obtained for each fish at the termination of the study when the fish were humanely euthanized (overdose of TMS). The length of study was determined by tank space and availability.

2.1.6 Tag retention

Each fish was examined for the presence of a tag at the time of mortality recovery or at the termination of the study. Tag location was identified and recorded.

2.1.7 Histological examination

The skin and underlying muscle where the incision was made, or would have been made in the case of non-incised fish, was obtained and preserved in 10% neutral buffered formalin for

histological examination. The tissues were decalcified using Cal-Ex11 (Fisher Scientific), processed using the Sakura Tissue-Tek VIP 5 (Somagen) and then stained with hematoxylin and eosin. Histopathology reading was done in a blinded manner such that the reader could not determine the treatment group of each slide.

The results of the histopathological examinations were designated as either normal, disorientation of muscle cells, or scale deposition/infection. Disorientation of the muscle cells was defined as any alteration in the muscle fibres that was not associated with a nerve or otherwise normal anatomical expectations. *Scale deposition* was defined as disorientation of the muscle cells with an external scale deposited inside of the muscle layers or penetrating into the peritoneal cavity. Infection was defined as disorientation of the muscle cells with associated changes, such as granulomas or bacteria.

2.1.8 Statistical analysis

All statistical analyses were performed using STATA (version 10) software (Statacorp, College Station, TX).

2.1.8.1 Weight, tag retention and histological analysis

To evaluate final weight for the three treatment groups, the data were evaluated for normality, and transformation of the data was considered. A log transformation was used to compare the final weights across the three treatment groups and between sexes. The proportion of tags retained (and its exact 95% binomial confidence interval) was computed. The histology results

were analyzed by cross-tabulation with treatment group and a Chi-Square Test applied to the table.

2.1.8.2 Survival analysis

The mortality data collected in this study consisted of the time until death of the fish. The entire population was censored (lethal sample) at 8 weeks. Sex was not recorded for all mortalities, and so this was not included in the survival analysis. Survival analysis in this study was performed using a Cox proportional hazards model, which included group and weight as well as their interactions. The Cox proportional hazards model is based on the assumption that the hazard for the individual is a product of a baseline hazard along with treatment effects, which are assumed to remain constant over time (Dohoo *et al.,* 2009). The assumption of proportional hazards was evaluated, and it was determined that this assumption was not violated.

2.2 Results

2.2.1 Tag placement

PIT tag placement in this study was located in the approximate region to the left of midline and 2 mm cranial to the anal pore. The radiographic evidence immediately following tag placement showed the area where the PIT tag was ventral and caudal to the liver (Figure 2.3). At the termination of the study, each fish was dissected, and there was no evidence of tag migration.

2.2.2 Tag retention

The PIT tagged group of cod was followed for 8 weeks post-tagging and there was 100% (95% CI: 98.2% – 100%) retention of the tag during this study period. All tags were functional at the end of the study.

2.2.3 Histological examination

Histological results in the control group consisted of 98.4% (95% CI: 91.4% – 99.9%) being classified as normal (Figure 2.4) and 1.6% (95% CI: 0.04% – 8.5%) being classified as scale deposition/infection (Figure 2.5). The results in the incision-only group consisted of 50.6% (95% CI: 39.4% – 61.8%) normal sections, 44.6% (95% CI: 33.7% – 55.9%) disorientation of the muscle fibres and 4.8% (95% CI: 1.3% – 11.9%) scale deposition/infection. For the PIT tag group, results consisted of 51.1% (95% CI: 40.2% – 61.9%) normal, 36.4% (95% CI: 26.4% – 47.3%) disorientation of the muscle fibres and 12.5% (95% CI: 6.4% – 21.2%) scale deposition/infection. The PIT tag and incision-only groups were statistically significantly different from the control group ($p<0.001$) but not statistically different from each other ($p=0.166$).

2.2.4 Weights

Final weights obtained for the control, incision-only and PIT tag groups averaged 38 g, 39 g and 44 g, respectively (Table 2.1). There was a statistical difference between the three groups, with the PIT tag group being heavier than the other two ($p=0.029$). The initial weight was recorded and there was no statistical difference among the three groups ($p=0.87$). There was a significant difference in weight between males and females for the study ($p=0.005$). Females had a mean weight of 42.4 g while the males had a mean weight of 35.8 g. The ratio of male-to-female in the

control, incision-only and PIT tag groups at the end of the study was 43.6% (30.3 – 57.7), 66.1% (52.2 – 78.2) and 58.5% (44.1 – 71.9), respectively.

2.2.5 Survival analysis

The days at risk for the study population comprised of 58 days, with all survivors being censored on day 58 by humane euthanasia. The results of the survival analysis showed no difference in survival among the treatment groups (p=0.904) (Table 2.2). The Kaplan-Meier survival graph (Figure 2.6) shows survival over the course of the study by treatment group.

2.3 Discussion

2.3.1 PIT tag placement and retention

In Atlantic cod, the tag placement location, left of midline and 2 mm cranial to the anal pore, resulted in the tag being retained ventral and caudal to the liver. Tag retention for this study was 100%, which were similar to the results in Atlantic salmon reported by Gries and Letcher (2002). Gries and Letcher (2002) reported tag retention of 99.8% after placing the tags through a scalpel incision and not suturing the wound post-tagging. Other studies evaluating PIT tag retention in various species report tag retention from 85% to 100% (Hopko *et al.*, 2010). Larger incisions are thought to increase the possibility of the tag being lost through the wound. Therefore, care must be taken to ensure the smallest incision/puncture wound is used (Gries and Letcher, 2002). Gheorghiu *et al.* (2010) designed a study to evaluate encapsulation of the tag 8 – 12 months post-placement. That study demonstrated that all PIT tags implanted in brown trout were encapsulated and approximately 40% had migrated to other regions. Our study did not evaluate encapsulation or migration of the PIT tag over the long term. The final

location of the tag was noted to be within the region of intended placement within the intraperitoneal space, and no migration or adhesions had occurred after 8 weeks. To fully evaluate migration, encapsulation or adhesions post-tagging, a longer duration study (from tag placement through to harvest) is recommended with each group being evaluated in triplicate.

If PIT tags are to be used for surveillance or research in fish destined to be harvested for human consumption, care must be taken to ensure that each fish tag is found and removed. Discarding fish that are not found to have a PIT tag at the time of harvest is a good practice to ensure that dormant tags do not remain inside the fish. In addition, some fish will consume smaller fish within a tank or cage, and so multiple PIT tags can be found in the stomach or intestine. To ensure that this is not an issue, it is recommended that the fish be scanned again after the PIT tag has been removed to look for other tags that might be inside of the gut. Some researchers will recommend that fish sold for harvest are filleted and not sold as Head On Gutted (HOG). Taking these precautions will provide some assurances that fish entering the market will not have PIT tags left inside them.

2.3.2 Histological findings

The histological findings indicate that the control group had normal skin and muscle compared to the other two groups. Both the incision-only and PIT tag groups had disorientation in the muscle fibres, and the PIT tag group had evidence of scale deposition/infection. These findings were not unexpected and suggest that adequate healing had not occurred at 10˚C (mean temp) by 8 weeks post-tagging/incision. This time frame for healing is longer than other reports in the

literature. For example, when seabream were evaluated post-tagging, a mean healing time of 20 days post-tagging was reported (Navarro *et al.*, 2006). However, juvenile pikeperch were not completely healed by 3 weeks post-tagging (Hopko et *al.*, 2006). Reasons for the difference in healing time may be related to immune response, age of animal, species or the water temperature. For the present study, histopathology was used to evaluate the skin tissues post-tagging; this is typically not the case in most other studies. If we were to characterize healing by visual inspection alone, the results may have been similar to other studies. To further evaluate healing and post-treatment complications over time, a longer-term study would be required, although costly and time consuming. In addition, visual inspection of the wound using standardized parameters would be an asset and therefore comparability to other studies would be possible.

2.3.3 Weight

Weight analysis was performed on 256 of the 264 fish, and not recorded for 8 fish. Final weight was significantly different across the treatment groups, with the PIT tag group weighing more than the other two groups. These results are inconsistent with other studies (Navarro *et al.,* 2006; Lee *et al.,* 2009) that show no difference in weight in other species. In fact, Hopko *et al*. (2006) states that it is expected that the tagging procedure will result in decreased or slowed growth for one week post-tagging, but that compensatory growth will result in no difference within a few weeks. The reason for the higher weight in the PIT tag group may include the weight of the PIT tag and scar tissue/inflammation associated with the tagging. The actual weight of the PIT tag is very small (~0.06 g), and so this alone could not be the sole cause of this weight difference. The difference in final weight between females and males was 6.6 g, with females weighing more than males. This finding is not unexpected as female cod tend to weigh

more than male cod. The PIT tag group did not have the highest proportion of females, and so this could account for the increased weight. Another consideration is that handling occurred during PIT tag placement (palpation of the ventral surface of the fish after tag placement). Still, there is no obvious reason as to why this would result in a difference in growth. In fact, studies have shown that stress due to handling will decrease, not increase, growth (McCormick *et al.*, 1998; Wilkinson *et al.*, 2006). Stress of handling and PIT tag placement should have affected all groups in the study and should have resulted in decreased growth. If this study had been conducted in triplicate and over a longer duration, this finding may have been found to be a result of tank effect, or it may have disappeared completely.

2.3.4 Survival analysis

PIT tagging had no effect on survival when compared to the other two groups (control and incision-only). These results are consistent with studies performed in other species (Navarro *et al.,* 2006; Lee *et al.,* 2009). Survival post-tagging is one of the biggest concerns that industry had when we were considering using commercial fish in a study design that required PIT tagging. To be able to show that survival is not affected by the tagging procedure and that these results are consistent with other findings is an important piece of information to document. The eight-week duration of this study did provide sufficient time to allow secondary infections that may have increased mortality to become apparent.

2.4 Concluding remarks

This study indicates that PIT tag placement in Atlantic cod (~6 – 18 g) located left of midline and 2 mm cranial to the anal pore into the intraperitoneal cavity is not associated with adverse

effects. No negative impacts on weight and/or survival were detected during this 8-week trial. Intraperitoneal PIT tags require minimal equipment to implant. When placed using a scalpel blade, the fish had 100% tag retention. PIT tags represent a useful tool in identifying individual Atlantic cod for the purposes of such activities as aquaculture and ecological research, broodstock identification and disease surveillance.

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Figure 2.1: Incision location in Atlantic cod 6 – 18 g

Figure 2.2: PIT placement in Atlantic cod 6 – 18 g

Figure 2.3: Radiograph showing PIT tag post-tagging taken immediately following placement in Atlantic cod 6 – 18 g

Figure 2.4: Histology showing Atlantic cod skin when no PIT tag has been placed

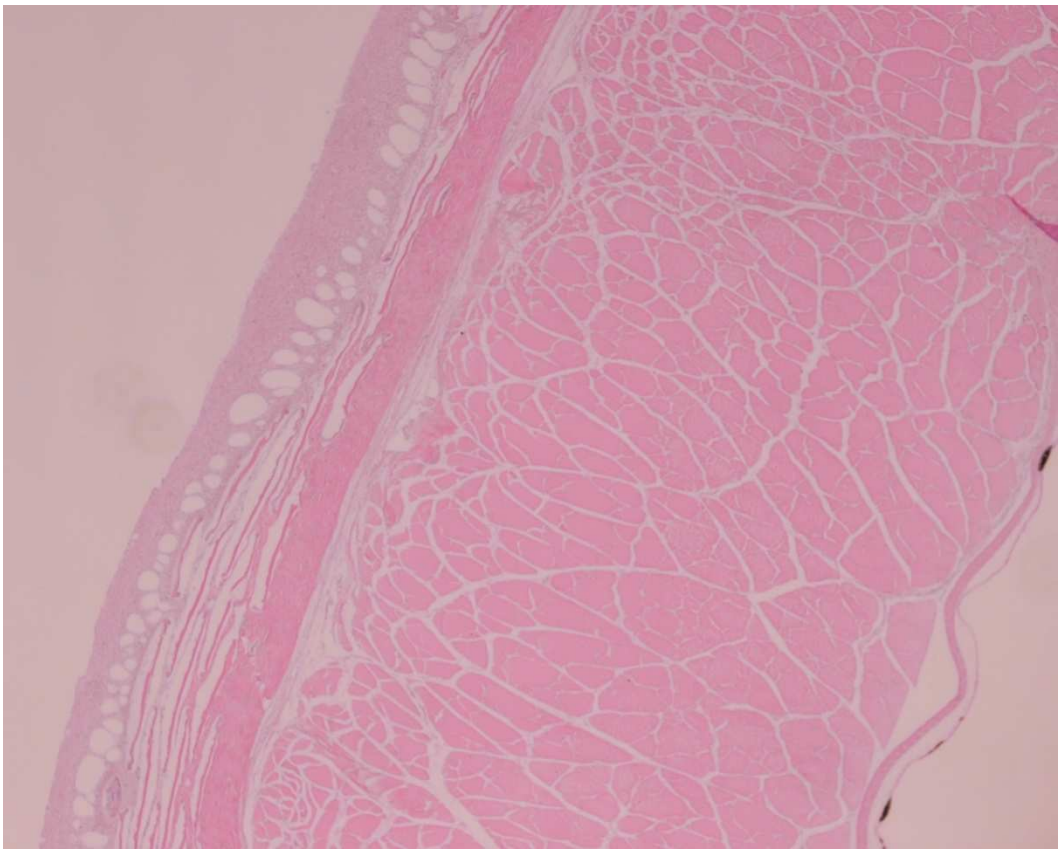

Figure 2.5: Histology showing skin after PIT tag placement. This illustrates disorientation of muscle fibres/inflammation.

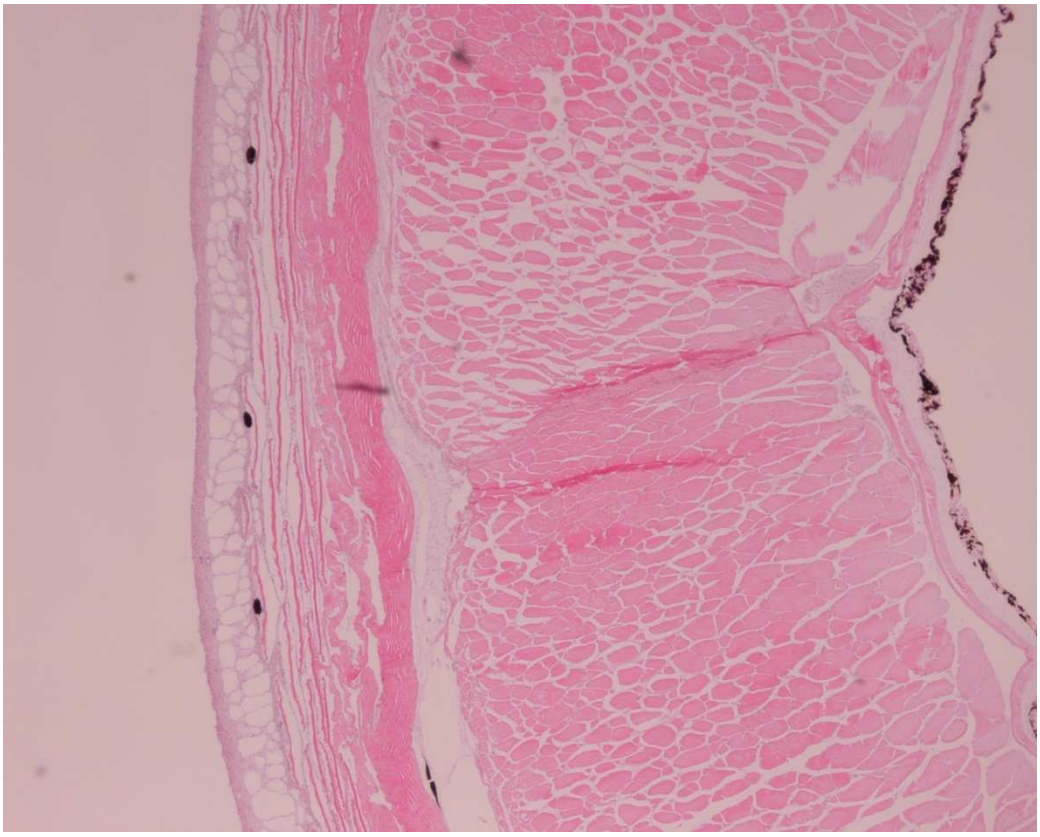

Figure 2.6: Kaplan-Meier estimates of the survivor function to PIT tag placement for 3 treatment groups in a study of juvenile cod.

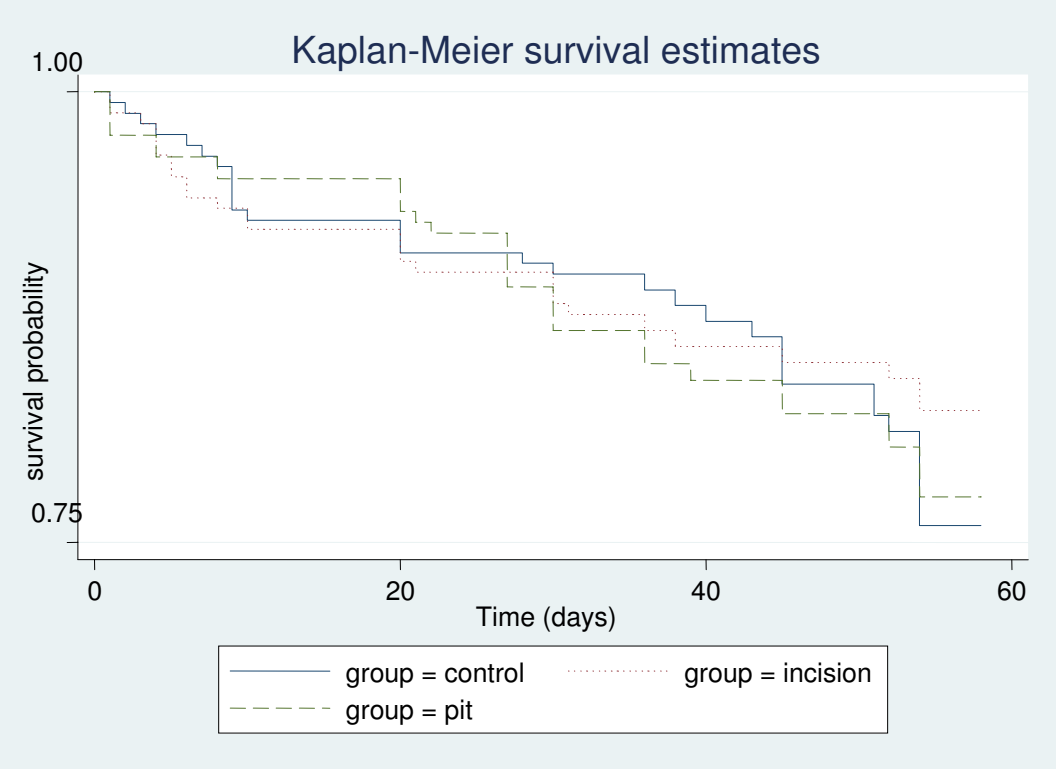

Table 2.1: Final weights of Atlantic cod survivors 58 days after PIT tag placement.

Treatment Group	Mean Weight (g)	Standard Deviation (g)	Median (g)
Control	38.4	17.9	36.0
Incision-only	38.7	18.6	36.0
PIT tag	44.0	20.5	43.5

Table 2.2: Hazard ratio of mortality in PIT tagged and incision-only compared with control groups in Atlantic cod.

Risk Factor	Hazard Ratio	P-value	95% CI[1]
Treatment Group		0.90	
Control	1		
Incision-Only	0.95	0.70	0.73-1.23
PIT tag	1.00	0.99	0.78-1.29

[1]95% Confidence Interval

Chapter 3: Survival analysis describing a nodavirus outbreak in Atlantic cod (*Gadus morhua*): effect of vaccine, temperature and dissolved oxygen over a 51-day period

3.0 Introduction

Atlantic Cod, *Gadus morhua*, aquaculture has faced many challenges worldwide (Bricknell *et al*., 2006). Emerging aquaculture species are usually poorly understood, as are their pathogens and diseases. When discovered, their pathogenesis, diagnostics, mitigation and treatment present challenges requiring further research and development (Bricknell *et al*., 2006). Some examples of the challenges that the cod aquaculture industry has faced are early maturation, proper nutrition and diseases, such as *Francisella* sp. (Bakkemo *et al.*, 2011) and nodavirus. Evidence-based medicine integrates clinical expertise and research to formulate or adjust clinical decisions for a particular pathogen or mitigation strategy (Sackett, 1996). Field trials and outbreak investigations contribute valuable evidence to the decision-making process.

One of the most significant cod viral pathogens that have emerged is nodavirus. Nodavirus causing the disease viral nervous necrosis (VNN), also known as viral encephalopathy and retinopathy (VER) or fish encephalitis, can affect over 30 species of marine finfish globally (Munday *et al.* 2002, Office International des Epizooties, 2003). High mortality and morbidity in larval and juvenile Atlantic cod has been reported in North America (Johnson *et al.,* 2002; Gagné *et al.,* 2004), Norway (Patel *et al.,* 2007) and Scotland (Starkey *et al.,* 2001). Nodavirus (Nodaviridae: Betanodavirus) is small (25-30 nm) and non-enveloped, and its genome is composed of two single-stranded positive-sense RNAs (Johnson *et al.,* 2002; OIE, 2003). Horizontal transmission of nodavirus is well documented in Atlantic cod (Munday *et al.,* 2002). Vertical transmission of nodavirus has been documented in other species, and it is assumed to

possibly occur in Atlantic cod (Samuelsen *et al.,* 2006). Clinical signs of VNN include looping or spiral swimming, dark colour, inappetance, lethargy and disorientation in the water column (Starkey *et al.,* 2001; Johnson *et al.,* 2002; Munday *et al.,* 2002). Patel *et al.* (2007) reported 15% mortality in 5 to 24 gram juvenile cod at a marine cage site in Norway. Starkey *et al.* (2001) observed 2% mortality in 1.5 to 3.5 gram hatchery-reared juvenile cod over a 3-month period in the UK. Johnson *et al.* (2002) reported 31% mortality in juvenile cod with a mean weight of 3.0 g over a 35-day period housed in tanks at 16 – 17°C at a research facility in Nova Scotia, Canada.

Standard diagnostic methods for nodavirus involve histopathology, RT-PCR (reverse transcriptase polymerase chain reaction) and virus isolation using the Striped Snakehead cell line (SSN-1) or a cell clone of SSN-1 (E-11) (OIE 2003). Histopathological lesions consist of necrosis and vacuolation of the brain, spinal cord and retina (Starkey *et al.,* 2001; Johnson *et al.,* 2002; Office International des Epizooties, 2003). The histological lesions occurring in the anterior brain and spinal cord are more extensive than those observed in the posterior region (Munday, 2002).

The innate, or non-specific, pathway appears to be a particularly important piece in the immune response to pathogens in cod (Lange *et al.*, 2005). Nonetheless, the innate immune system in cod is not fully understood, and, while elements like natural antibodies are elevated in cod, there remains controversy about their importance in host defence (Whyte, 2007). Compared to other teleosts, such as Atlantic salmon (Salmo salar), the cod immune system does have high IgM levels (natural antibodies), yet these fish tend to generate low levels of specific antibodies in response to vaccination or exposure to a pathogen. Taken with high levels of phagocytic

neutrophils in the peripheral blood (Star *et al.*, 2011), these antibodies point to the role of innate defences in protection from infection. Indeed, Star *et al.* (2011) sequenced the whole genome of the Atlantic cod and found that they lack the genes for the major histocompatibility complex (MHC) II, CD4 and invariant chain (Ii). These findings would explain why cod produce low levels of antibodies in response to a pathogen, as cells bearing these markers are responsible for the humoral (antibody) adaptive response to T-dependent antigens like proteins — a major component of both vaccines and infectious agents. The other finding was that the cod immune system has additional MHC I molecules and unique Toll-like receptors (TLRs), Implicating perhaps a cytotoxic T cell response or innate immunity compensating for the lack of CD4 T cells and a vigorous humoral response. It is known that the innate system does play a role in the directing of the adaptive immune response (Bakkemo *et al.*, 2011), which in this case would likely be CD8 bearing lymphocytes. Despite the lack of MHC II and CD4, and with additional MHC I molecules and unique Toll-like receptors, cod do not appear to be immunocompromised in their natural environment. This suggests that the adaptive immune response in cod are initiated and regulated differently than other teleosts. These findings have been noted in other gadoids, as well. Therefore, the divergence of the adaptive immune response may have occurred long ago (Star *et al.,* 2011).

A commercially available vaccine is not available for use against nodavirus in cod aquaculture. However, producers still want to vaccinate their cod populations and will often use salmonid vaccines, applying them in a similar way to salmonid aquaculture production. Although this outbreak of nodavirus was not initiated as part of the original study, the randomized groups in an immersion vaccine (against vibriosis) field trial became an opportunity to evaluate non-

specific protection. Our immunological hypothesis was that vaccination with bacteria and associated bacteria antigens enhanced the cod's innate response, which translated into a degree of protection against a virus infection.

Nodavirus has been responsible for various mortality rates, which may have been influenced by environmental conditions such as warm water temperatures, poor nutrition or increased stress due to handling. Patel *et al.* (2007) reported that water temperatures reaching 18 to 19 °C were responsible for increased mortality during a nodavirus outbreak. Quantifying parameters such as temperature and oxygen, which are sometimes possible to manipulate during an outbreak, provides information that may be used to ultimately decrease mortality and morbidity during an outbreak. The objective of this study was to evaluate the impact of environmental factors, particularly water temperature and oxygen, on mortality in groups of vaccinated (against vibriosis) and non-vaccinated cod during a natural nodavirus outbreak.

3.1 Materials and methods

3.1.1 Clinical trial management

The trial was conducted at a research facility in Newfoundland and Labrador. Atlantic cod juveniles weighing 5 – 7 g were randomized using systematic random sampling into six tanks on August 16, 2005. Fish were kept at low densities and were maintained in a flow-through system. A dip vaccine (Vibrogen II, Novartis Animal Health) containing *Vibrio anguillarum*, serotypes O1 & O2 and *Vibrio ordalii* was applied to fish in three tanks on Day 0 (August 26, 2005). The tanks received vaccine or sham vaccine based on random allocation by drawing tank numbers out of a hat. The vaccine was mixed according to the manufacturer's instructions using a dilution with

seawater for the three treatment tanks. The fish were dipped out of the tanks into 4 Litre buckets containing, on average, 200 fish per bucket. The fish were then placed into a net and then dipped into an aerated bath with either the vaccine or the sham for 60 seconds. The three control tanks followed the same procedure using seawater only, as a placebo. The study personnel (involved in vaccine administration, data collection and fish handling/feeding) were blinded to the treatment allocation. Mortalities were removed from each tank and recorded twice daily.

3.1.2 Clinical samples

Health surveillance of the population was performed by removing moribund Atlantic cod at any point from Day 0 (August 26, 2005) until the termination of the study on Day 51 (Oct 15, 2005). Moribund fish were necropsied and diagnostic tests performed. Systematic random samples of the population were obtained on Days 0 (n= 50) and 51 (n= 50) for diagnostic testing. Bacterial cultures were performed using a kidney swab on BA (blood agar with 2% salt) and TSA (tryptic soy agar with salt). The BA plates, incubated at 15°C and 22°C, were checked daily for the first week and then held for observation another 4 weeks. The TSA plates were incubated at 22°C, checked daily and held for observation another 4 weeks. Virus isolation (VI) was performed using SSN-1 and RT-PCR. The tissues obtained for VI included kidney, heart, spleen, gill, brain and eye. Kidney, heart, spleen, pyloric cecea, gill, liver, brain and eye samples were also preserved in 10% buffered formalin and then prepared using H & E (Hematoxylin and Eosin) stain for histological examination.

3.1.3 Environmental variables

Daily records for each tank included mortality, water temperature (°C) and dissolved oxygen (%). The temperature and dissolved oxygen were recorded using a portable hand-held reader (Handy Portable DO meter, Oxyguard), which automatically compensates for atmospheric pressure and measures the partial pressure of oxygen in the water. The variables included in the analysis of the mortality patterns over time included the oxygen and temperature measured on each day, the vaccine group coded as a dichotomous variable, as well as the mean temperature and oxygen over a three-day period prior to each day. The last two variables were based on the hypothesis that sustained suboptimal conditions could increase stress in the fish and hence be correlated with increased mortality during a nodavirus outbreak. The average over three days was chosen based on a consideration of biological relevance and after exploring different duration averages, in particular 5- and 7- day averages.

3.1.4 Survival analysis

Two statistical models and approaches were used: a Cox proportional hazards model and a discrete time survival model (Dohoo *et al.,* 2009). The Cox model was used to assess the effects of vaccine and between-tank differences in temperature and oxygen on mortality. With temperature and oxygen specified as time-varying covariates, the between-day variations of temperature and oxygen are absorbed into the baseline hazard and, therefore, the Cox model only evaluates the within-day (between-tank) variations of temperature and oxygen. To account for clustering of fish in tanks, the effect of vaccine was assessed in the model with random tank effects (gamma shared frailty model). The effects of temperature and oxygen were assessed after adjusting for clustering at the combined tank-day level by robust variance estimation. The

assumption of proportional hazards was evaluated by tests based on scaled Schoenfeld residuals (Dohoo *et al.,* 2009).

A discrete-time survival analysis was used to assess the impact of the between-day variation of environmental variables on the daily mortalities in the six tanks (Singer and Willett, 2003). The modeling, of time (days at risk) represented a challenge: balancing an unrealistic assumption of time effects that were absent during a natural outbreak, with an overly flexible model of time that might eliminate the effects of environmental variables. A second-order polynomial (rather than a higher-order polynomial) was chosen to allow for a smoothly varying baseline hazard function of time. Discrete time survival analysis was implemented as a generalized linear model with cloglog link function, fixed effects of tanks, and standard errors adjusted by a Pearson overdispersion factor computed at the combined tank-day level (Dohoo *et al.,* 2009). Because a linear effect of the environmental predictors could not be given a biological interpretation, and because both linear and quadratic effects of the predictors were not supported by the data, the temperature, oxygen, 3-day temperature average and 3-day oxygen average were categorized into 4 categories with roughly equal proportions of values in the four categories for each predictor. In order to ensure that the effects of categorical predictors were not influenced substantially by the categorization, the analysis was repeated with classification 3 and 5 categories, and only predictors that showed significant and consistent effects in all categorizations were retained. Interactions between significant predictors were assessed, subject to the same condition of consistency across categorizations.

The significance level for retention in the statistical analysis was set at $P < 0.05$, and all analyses were carried out using Stata® (version 10) software (Stat Corp, College Station, TX).

3.2 Results

3.2.1 Study population and environment

The study included 10,749 Atlantic cod distributed roughly evenly in the six tanks (tank percentages: 15.9 – 16.8% of the total study population). By day 50, the overall cumulative mortality exceeded 60%, and the decision was taken to cull the population the next day. The mean and median days until mortality were 23.5 and 23 days, respectively (Table 3.1). The mean temperature and oxygen concentrations during the period were 10.7 °C (range: 9.2-12.9 °C) and 103.6% (range: 70 – 137%), respectively (Table 3.1). The daily temperatures were similar across tanks, but the oxygen concentrations were somewhat more variable (e.g., on 70% of the days, the maximal temperature and oxygen differences were at most 0.1 °C and 20%, respectively). The water temperature decreased markedly and stabilized at a lower level at around 25 days (Figure 3.1), most likely due to a thermocline inversion in the bay supplying the water to the hatchery. The oxygen concentrations showed a converse, but weaker, pattern (Figure 3.2).

3.2.2 Disease history

The Atlantic cod in this study were obtained from broodstock that were positive for nodavirus but which never exhibited any clinical signs of VNN. Starting on day 11 (September 6, 2005), clinical signs associated with nodavirus infection were observed in all tanks in the study population. The clinical signs in the juvenile population included abnormal swimming behaviour, with the weaker fish swimming in circles, on their sides, at the top of the water column or near

the drain at the bottom of the tank. When the abnormally swimming fish were physically stimulated, they would right themselves, actively swim away and then resume their abnormal swimming behaviour. The fish were dark in colour, but they remained on feed.

3.2.3 Diagnostic test results

Histopathological lesions revealed multifocal areas of retinal neuronal vacuolation and degeneration. The brain revealed marked fibro-histiocytic infiltration and vacuolation. All samples tested were positive for nodavirus using RT-PCR, and cytopathic effects were observed on cell culture using the SSN-1 cell line. Bacteriologic cultures did not exhibit any microbial growth on BA or TSA. Histologically, xenomas (associated with concomitant microsporidia infection) were observed in low frequency in some of the fish (n=23).

3.2.4 Cox proportional hazards model

In terms of both observed mortality and estimated hazard of mortality, the vaccinated tanks ranked 1st, 2nd and 4th among the six tanks, and the Cox model analysis showed a significant, protective vaccine effect: hazard ratio (HR) = 0.93 (95% CI: 0.87-0.99, P-value=0.03). The survival rates were similar in all tanks until about Day 20 (Figure 3.3), when mortality became more variable between both individual days and tanks (Figure 3.4). There was no statistical evidence of non-proportional hazards for tanks or vaccine groups. Between-tank variations in temperature and oxygen were not significantly associated with the hazard of mortality when the deviations from the daily means across all tanks were modelled by either a linear term or a categorical predictor.

3.2.5 Discrete time survival analysis

Due to the shift in both mortality patterns and the ranges of the environmental variables after the thermocline inversion, the analysis of the impact of temperature and oxygen was performed separately in two periods, prior to and after the thermocline inversion. On the basis of Figure 3.1, the start of the second temperature phase was determined to be at day 23, and, in order to avoid overlap between periods in the 3-day lagged variables, the analysis time was split into two periods days: 1-25 and 26-51.

In the first 25 days of the outbreak, only the temperature on the day of sampling was statistically significant, with the lowest hazard in the central temperature range (10.8-11.3 °C, Table 3.2).

For the last 26 days of the outbreak, effects were found for both temperature and oxygen variables (Table 3.3). The temperature effects indicated the lowest mortality during high temperatures, and a marginally significant (P=0.07) interaction between the temperature variables further indicated the lowest risk to be associated with high temperatures both three days prior to and on the day of sampling (not shown). The hazard was higher for elevated average oxygen concentrations three days prior to sampling. The strong fluctuation in mortality on days 22-30 and the seemingly cyclical patterns in mortality after day 30 (Figure 3.4) could not be readily linked to the values of the environmental variables, and both the final models exhibited a substantial overdispersion (estimated overdispersion factors of 9.6 and 11.0, respectively).

3.3 Discussion

To our knowledge, the effect of dip vaccine containing a bacterial antigen on survival during a viral outbreak in cod or any finfish has not been described previously. The samples obtained did not show evidence of any concurrent infectious disease, and the clinical signs and progression of the Nodavirus infection were consistent with previously reported patterns in the literature (Starkey *et al.,* 2001; Johnson *et al.,* 2002; Patel *et al.,* 2007).

The vaccine showed evidence of protection, which was most apparent after approximately 20 days. This lag response is expected after vaccination in animals or fish where the outcome is generation of an adaptive response manifested in the production of antibodies. In salmonid aquaculture, each vaccine requires a certain length of time (degree days), based on temperature, before the fish are considered protected. The lack of MHC II and CD4 in cod would indicate that the salmonid adaptive humoral immune response to a vaccine is not involved with the protection seen here and, hence, we are assigning protection to innate immunity. Strengthening this supposition is the fact that there are no specific antigens from nodavirus in the vaccine preparation, and so involvement of MHC I restricted CD8 T cells seems unlikely. It is curious, then, why the lag between vaccination and protection occurs. We know little about the kinetics of activation of the innate immune mechanisms in cod.

The effect of vaccine was not entirely consistent across tanks, and, given the small number of tanks, it would be advisable to confirm the findings in a study with additional tanks or study groups. The dip vaccine, containing bacteria and their antigens, may be protective in a viral

outbreak. It is known that the innate immune system will direct the adaptive immune response and that the LPS components of bacteria (as was included in the vaccine used) will result in a non-specific response to vaccination. Although this vaccine did not contain an adjuvant, the innate immune system in cod can be stimulated by an adjuvant alone (Magnadóttir *et al.,* 2001). Bacterial components themselves are known to act as adjuvants. Therefore, a likely hypothesis is that vaccination with bacterial vaccines or non-bacterial vaccines containing an adjuvant may result in an innate immune response post vaccination that may be beneficial in cod if they are expected to encounter a pathogen challenge.

Normally when cod are transferred to the marine cage site, they will be challenged by pathogens in the wild. Therefore, vaccination — to elicit a specific response prior to transfer — may be warranted. The most common regime for salmonids in Eastern Canada consists of an intraperitoneal vaccination about 440 degree days prior to transfer. Vaccination using a water-based dip vaccine in cod prior to marine water transfer may have some benefits, but the time prior to transfer and the vaccine's efficacy over time must be considered. Likewise, elicitation of an innate response may provide protection. Handling cod shortly before transfer may stress the fish and translate into any benefit to vaccination being lost. Future studies to evaluate the required degree days until protection and how long the vaccine or immunostimulant will be protective for should be considered to be able to more critically evaluate this question. The cost of the vaccine, labour required and effect on the animals must all be considered, as well. Many producers will vaccinate, and, as nothing negative may have happened in the past, they will continue this practice because they do not see the practice as harmful — if it helps, it would have been worth it. However, if the regime could be established for cod vaccination, the

benefits could be enhanced. Future studies should include a measure of immune response from the cod to help determine the effectiveness of the vaccine. If this practice is deemed unnecessary, then this activity could be halted, saving time, money and animal handling.

Every year in fall, the established thermocline in the bay supplying the water to this hatchery inverts, causing the incoming water into the facility to cool suddenly and rapidly. This would explain the difference in water temperature between the first 25 days and the last 26 days of the study period. Both before and after the thermocline inversion, temperature showed an impact on mortality. However, the direction of the effects was opposite: during the first 25 days, the temperature range was 9.8°C to 12.9°C, with the middle range of temperatures being most beneficial (10.8°C to 11.3°C). These warmer water temperatures were present during the time when nodavirus was detected in the population. Patel et al (2007) showed that increased water temperatures were highly correlated with nodavirus outbreaks in Norway. In the last 26 days, the temperature range was 9.2°C to 11.1°C, with the warmer water temperatures being protective. This finding is consistent with reports that sick wild cod will move into warmer water temperatures temporarily. It is thought that the specific or acquired immune system and some components of the innate system are more efficient at these higher temperatures than at lower ones as long as the duration is short and it is not too much above the optimum temperature (personal communication, Magnadóttir, 2007). It appears that juvenile Atlantic cod, during a nodavirus outbreak, seek a preferred environment with respect to water temperature. High oxygen concentrations were associated with higher mortality after the inversion. Sub-optimal dissolved oxygen has been associated with increased stress, morbidity and mortality (Howell

and Baynes, 2004). These effects will be increased or more evident during times of stress such as a nodavirus outbreak.

The large and unexplained variation in mortality between days and tanks indicates that, despite the overall mortality trends associated with temperature and oxygen, the day-to-day variation in mortality is largely unexplained and possibly related to other, potentially immeasurable environmental factors or to infection dynamics in the population.

3.4 Concluding remarks

In conclusion, the effect of bacterial antigen dip vaccines may provide some protection during a nodavirus outbreak. These findings warrant further investigation into vaccine use in Atlantic cod and suggest that innate immunity may play a significant role in the protection of Atlantic cod. Monitoring and maintaining optimal temperature and oxygen during a disease outbreak are critical in decreasing mortality. Husbandry practices that optimize the environment will be important to managing disease mortality in cod aquaculture.

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Table 3.1: Descriptive statistics of environmental parameters over the 51-day period

Variable	Mean	St. Dev.[3]	Min	Max
Dissolved Oxygen (%)	103.6	11.59	70	137
Temperature (°C)	10.7	0.86	9.2	12.9
Ave Temp3[1]	10.8	0.75	9.7	12.3
Ave Ox3[2]	103.2	8.96	80	135

[1]Ave Temp3 = Average 3-day temperature

[2]Ave Ox3 = Average 3-day dissolved oxygen

[3]St. Dev. = Standard deviation

Table 3.2: Effects of environmental variables on the hazard of mortality during the first 25 days of nodavirus outbreak in hatchery-raised juvenile Atlantic cod, estimated by discrete time survival analysis

Risk Factor	Hazard Ratio	P-value	95% CI[1]
Temperature			0.008
9.8-10.8	1	-	-
10.8-11.3	0.55	0.004	0.36-0.83
11.3-11.9	0.80	0.31	0.51-1.24
11.9-12.9	0.89	0.57	0.60-1.32

[1]confidence interval

Table 3.3: Effect of environmental variables on the hazard of mortality during the last 25 days of nodavirus outbreak in hatchery-raised juvenile Atlantic cod, estimated by discrete time survival analysis

Risk Factor	Hazard Ratio	P-value	95% Confidence Interval
Temperature			<0.001
9.2-9.8	1	-	-
9.8-10.1	0.78	0.20	0.53-1.14
10.1-10.4	0.92	0.68	0.62-1.36
10.4-11.1	0.38	<0.001	0.24-0.61
Ave 3-day Temperature			0.020
9.73-9.97	1	-	-
9.97-10.08	0.88	0.46	0.63-1.24
10.08-10.33	0.69	0.08	0.46-1.04
10.33-10.87	0.52	0.003	0.33-0.80
Ave 3-day dissolved Oxygen			0.002
86.3-103.8%	1	-	-
103.8-106.3%	1.30	0.31	0.78-2.17
106.3-109.0%	1.62	0.05	0.99-2.65
109.0-118.0%	2.19	0.001	1.38-3.48

Figure 3.1: Mean water temperature through the study period

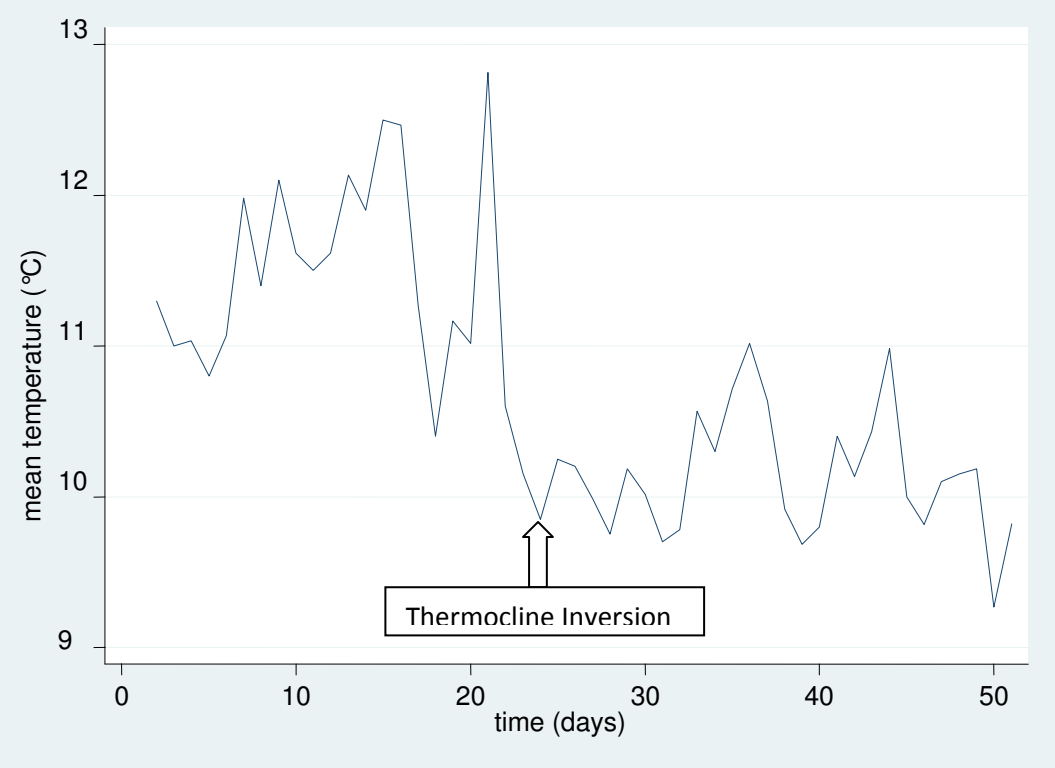


Figure 3.2: Mean dissolved oxygen (percent) through the study period

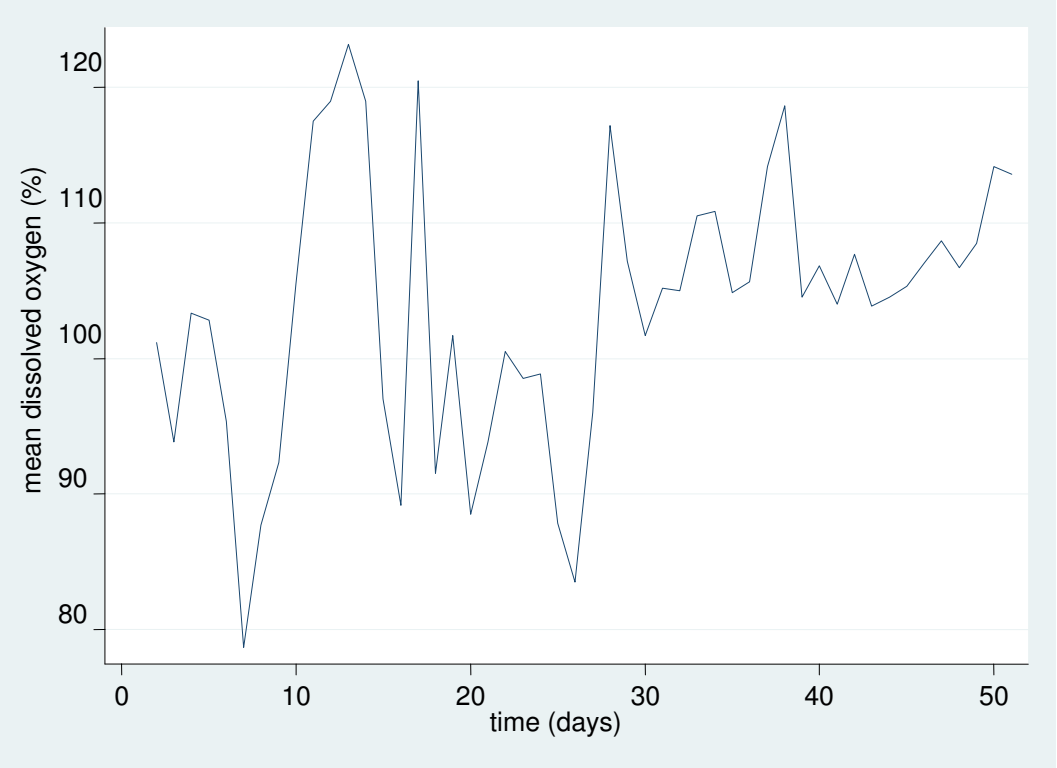
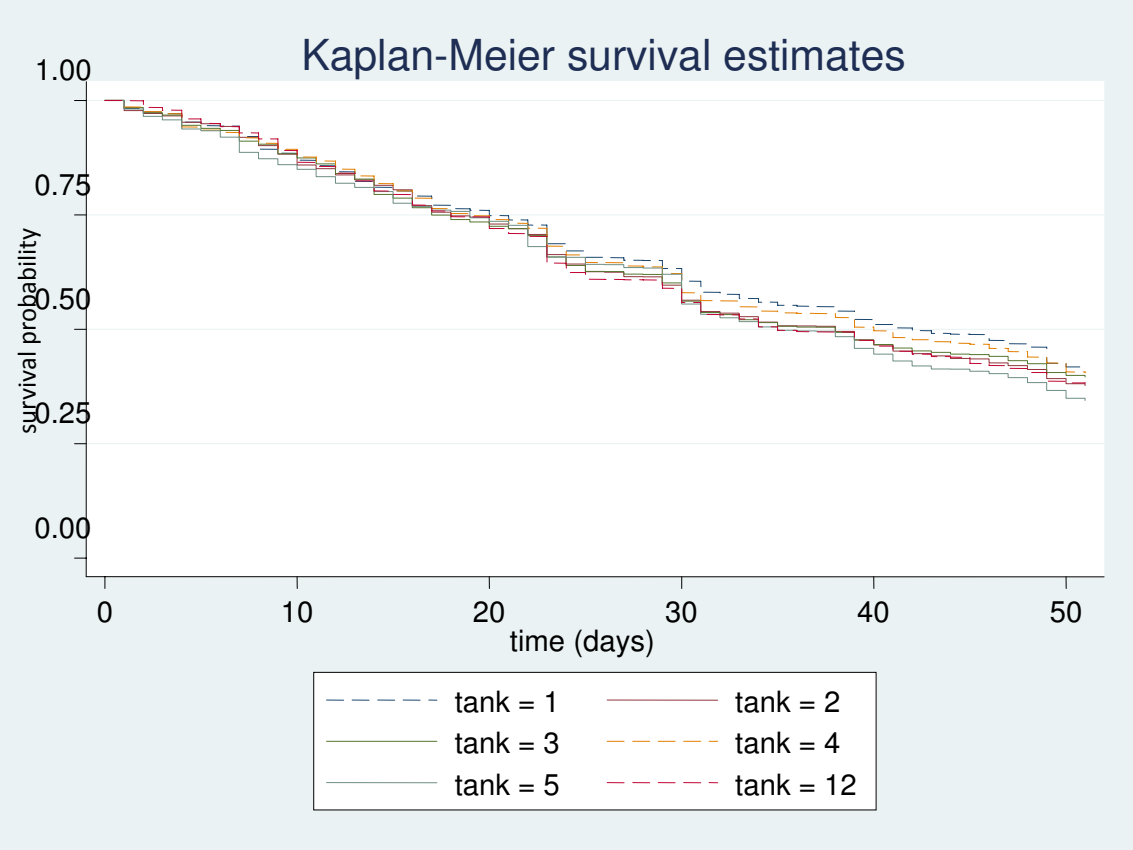
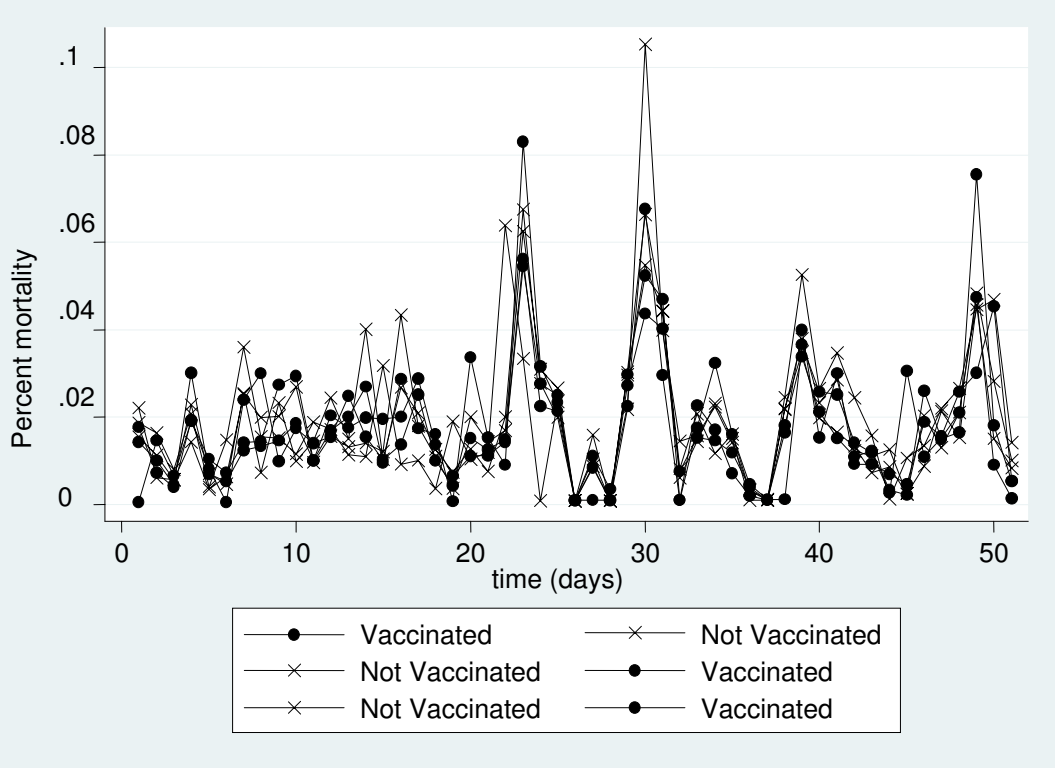


Figure 3.3: Kaplan-Meier survival rates in the six tanks over the 51-day period



Note: dashed line indicates a vaccinated tank.

Figure 3.4: Daily mortality (%) in the six tanks



Chapter 4: The effect of the SuperSmolt^{TM} process on physiological characteristics, growth and survival in Atlantic salmon (*Salmo salar*).

4.0 Introduction

As a diadromous species, wild Atlantic salmon (*Salmo salar*) spawn and live their early life stages in freshwater and then, if possible, migrate to seawater for their adult life stages. They then return to their natal river for spawning. The freshwater hypoosmotic environment requires physiological capability to maintain a static osmotic state that is more concentrated within the fish than its surrounding environment (Smith, 1993). While in freshwater, fish actively retain salts (cations) through their gills. In contrast, the saltwater environment is hyperosmotic, and salmon must undergo physiological changes to allow them to survive under completely different osmotic pressures (Björnsson *et al.,* 2011). Fish drink saltwater while in the ocean, and the gills extrude monovalent ions while the kidney extrudes divalent ions. Smoltification is the term used to describe the entire process of physiological alterations that enable the fish to survive the transition from freshwater to seawater environments.

The process of smoltification, which can take months to complete, is initiated by environmental cues such as photoperiod and temperature (McCormick and Björnsson, 1994; Nilsen *et al.*, 2008; Bjornson *et al.,* 2011). Although photoperiod is the most important determinant, temperature plays a substantial modifying role, as well (Wedemeyer, 1996; McCormick *et al.*, 2000). This complex procedure is driven by the endocrine system and involves morphological, biochemical, physiological and behavioural changes to the fish undergoing it. For example, the caudal peduncle is elongated to aid in swimming, and hemoglobin isoforms are changed to increase oxygen-carrying capacity. During the process, the fish lose parr markings and become a uniform

silver colour with marginal fin darkening, which may assist in predator avoidance in open water (Wedemeyer, 1996; Nilsen *et al.,* 2008; Bjornson *et al.,* 2011).

The endocrine profile during smoltification in Atlantic salmon is well documented. The key changes involved are growth hormone (GH), insulin-like growth factor, cortisol, thyroid hormones and prolactin. Cortisol and GH increase during smoltification and stimulate the development of brachial chloride cells and intestinal osmoregulatory function (Bjornson *et al.,* 2011). Elevated thyroid hormones are thought to be involved in the colour and behavioural changes observed during smoltification. Prolactin, which decreases during the smoltification process, is thought to reduce the normal inhibition of GH.

In an aquaculture setting, the fish are prepared for sea transfer in the hatchery by altering day length and temperature to optimize the physiological smolt window. Manipulation of this physiological smolt window enables transfer at times of the year not normally observed in nature, thus producing harvest-sized fish year round. Photoperiod manipulation and temperature control have been the primary methods used for adjusting the timing of smolt transfer. If the fish are not transferred during this physiological smolt window, many processes will reverse through a process called desmoltification (Björnsson *et al.,* 2011): The fish lose the ability to hypo-osmoregulate. GH appears to play a role in this desmoltification, or parr-reversion (Wedemeyer, 1996; Björnsson et al., 2011). S1 populations are defined as salmon smolt transferred from the freshwater hatchery to seawater in their first natural opportunity, which occurs in the spring (i.e., about 16 – 18 months after fertilization of eggs). S0 populations are defined as salmon smolt transferred to the marine site in the fall after one summer of

growth in freshwater (i.e., approximately 12 months after fertilization — approximately 6 months earlier than S1 transfers) (Smith, 1993).

SuperSmolt™ (currently supplied by Europharma Inc. [British Columbia, Canada], previously supplied by MariCal) claims to control osmoregulation in Atlantic salmon (*Salmo salar*) smolt to provide the salmon farming industry with increased flexibility in managing the smolt transfer period. Some additional company claims attributed to the use of SuperSmolt™ include 1) reducing a standard S0 size earlier; 2) generating larger S0 fish; 3) facilitating a synchronized smolt schedule; 4) increased fish survival post-transfer; and 5) decreased risk of disease during this period (Europharma, 2012). The SuperSmolt™ process involves an "all-natural" water treatment and special formulated feed. Despite the apparent health management claims, SuperSmolt™ is not considered a drug in Canada (i.e., a product affecting the health of animals). The cost for implementing the SuperSmolt™ process adds to the overall cost of production while claiming to reduce the overall productivity losses. Farm managers and their veterinarians require valid third-party clinical trials to provide evidence for decision-making regarding the benefits of products and procedures on health and productivity. The objectives of this study were to evaluate the clinical effects of SuperSmolt™ on Atlantic salmon smolt in the immediate transfer period and over the longer term. To evaluate the short-term effects of SuperSmolt™, biological parameters (ATPase and Osmolality) were measured before and after exposure separately on S1 and S0 populations. To evaluate the longer-term effects of SuperSmolt™, growth and survival were assessed in the S0 population through 262 days after the onset of exposure to SuperSmolt™.

4.1 Materials and methods

4.1.1 Study populations

This study was comprised of two different populations based on when they were transferred to the marine cage site after the hatchery stage. Monitoring, for the purposes of the study, began at the time of PIT tagging. The first study population was comprised of 4,525 S1 Atlantic salmon. Monitoring of this population was discontinued due to production decisions at the time of transfer, and 194 of the fish were lethally sampled for further physiological assessments. This population of fish will be referred to as the study 1 (S1) population here after. The second population consisted of 4,299 S0 Atlantic salmon. This population was followed for 262 days after starting SuperSmolt™ (exposure) in the hatchery (i.e.,228 days post-transfer to seawater) and will be referred to as study 2 (S0) population throughout the following discussion.

Both the study 1 (S1) and study 2 (S0) populations had PIT (Passive Integrated Transponder) tags (AVID, Alberta, Canada) implanted into the intraperitoneal space at 21 and 13 days, respectively, prior to the exposure (SuperSmolt™ treatment/procedure). The PIT tag, an internal microchip 12-14mm in length, 2mm in diameter and weighing 0.1 g, has a biocompatible glass encapsulated antenna copper coil that remains dormant until the scanner activates it with an electromagnetic field. The microchip generates an alphanumeric code unique to each individual fish enabling repeated measurements for the duration of the study.

4.1.1.1 Study 1 (S1)

This study included 4,525 S1 Atlantic salmon, previously PIT tagged and separated into two tanks for treatment with SuperSmolt™ (exposure) or no exposure (control). Prior to separation

of the groups, simple random sampling was performed to obtain a Time 0 (pre-exposed) sample of 85 fish. Fish were then randomly allocated into treatment (n=2,299, 1 tank) or control tanks (n=2,226, 1 tank). The allocation was achieved using a computer program to randomly assign the PIT tags to one of two treatment groups (exposure and control groups). Fish were taken from the same tank, anesthetized with Tricaine Methane Sulfonate (TMS, Syndel International, Vancouver, Canada), scanned for PIT tag identifications, weights and lengths obtained, and then placed into the appropriate study tanks based on the PIT tag number. By necessity (water treatment component), the control and exposure groups were maintained in separate tanks until the day of transfer. At time of transfer, systematic random sampling was performed to obtain samples from both the exposed (n=51) and control (n=58) groups. All remaining S1 fish were transferred to the same marine cage, and no further sampling occurred due to production decisions at the time of transfer.

4.1.1.2 Study 2 (S0)

The study 2 (S0) population was made up of 4,299 PIT tagged S0 Atlantic salmon. Fish were assigned to two tanks using a similar procedure as the Study 1 (S0) population with 2,147 in the control tank and 2,151 in the exposed tank. At the time of random allocation, a random sample of pre-exposed fish was obtained (n=69) for physiological assessment. As with the Study 1 (S1) population, control and exposure groups were maintained in separate tanks until the day of transfer. At the time of transfer, systematic random sampling was performed to obtain samples from both the exposure (n=82) and control (n=74) groups. All remaining fish were transferred to the same marine cage at a commercial production site and followed until 262 days after the onset of exposure to SuperSmolt^{TM}.

4.1.2 Treatment exposure

The treated groups in both study 1 (S1) and study 2 (S0) populations underwent similar SuperSmolt™ procedures. This required that the fish be fed a specially formulated SuperSmolt™ Xcelerator diet until 6 days prior to transfer when they were switched to Xcelerator Plus diet for 4 days. The fish were then taken off feed 2 days prior to transfer, as per normal industry standards, to facilitate the most effective transfer. To adjust the external environment, as per SuperSmolt™ protocol, calcium ($CaCl_2$) and magnesium ($MgCl_2$) were added to a mixing tank, allowed to dissolve and then distributed to the tanks. This mixture was added to the tanks twice daily. According to SuperSmolt™ recommended procedures, the farmer took conductivity readings (a measure of the ability of water to pass an electrical current) twice daily using a handheld electrical conductivity meter (supplied by MariCal). This was performed to ensure that the water conductivity was between 900 and 1,100 uM (expected range for flowthrough conditions as recommended by MariCal). S0 populations were also exposed to 24-hour light as per normal hatchery routine. The entire SuperSmolt™ process took approximately 30 days to complete, during the immediate period prior to smolt transfer to the marine cage site.

4.1.3 Health assessments

The pre-exposed samples for both study 1 (S1) and study 2 (S0) populations were obtained at the time of treatment allocation. For each sample, the fish were necropsied and further evaluated for selected salmonid pathogens. Bacterial cultures were performed using a kidney swab on BA (Blood Agar, no salt), TSA (Trypticase Soy Agar) and SKDM (Selective Kidney Disease Media). The BA plates, incubated at 15°C and 22°C, were checked daily for the first week and then weekly and held for a total of 4 weeks. The SKDM plates, incubated at 15°C, were checked

weekly for 12 weeks. Plates with colonies on any media were submitted to Aquatic Diagnostic Services at the Atlantic Veterinary College for species identification.

Virology testing was performed using inoculation of CHSE (Chinook Salmon Embryo) and SHK (Salmon Head Kidney) cell lines. The tissues obtained for virology evaluation were kidney, heart, spleen, and gill in pools of five fish each. Kidney, heart, spleen, pyloric cecae, gill and liver samples were preserved in 10% buffered formalin and then stained with H & E (hematoxylin and eosin) stain for histological examination. The study 2 (S0) population was monitored with mortality dives at least weekly throughout marine cage production until 228 days after smolt transfer. At the end of the follow-up period (day 228), a random sample (using simple random sampling by PIT tag number) of 50 fish were subjected to the same disease screening procedures as described earlier.

4.1.4 Osmolality samples

Each fish was euthanized by using percussive cranial stunning, and a pre-heparinized syringe and needle (1cc syringe, 25 gauge needle) was used to immediately obtain a blood sample from the caudal vein. Blood was then transferred to a microtube and separated using a Galaxy Mini Centrifuge 37000-700 (Ontario, Canada) at 6,000 RPM for 2 minutes. Plasma was extracted and placed into another microtube by gentle pipeting and maintained at $4^{o}C$ until analysis. Plasma osmolality was then measured by freezing point depression osmometry (Model 2020, Advanced Instruments, Massachusetts, USA).

4.1.5 Gill Adenosinetriphosphatase enzyme activity (ATPase) in gill samples

ATPase samples are used to determine the Na+ K+ ATPase activity in the gill. This was used as an indicator of the smoltification process. Using the same fish as described for osmolality samples, a gill tissue sample was obtained from fish in a right lateral recumbent position. The second gill arch was isolated, and gill filaments (approximately 2mm x 2mm) were removed using scissors and placed in SEI buffer solution (150 mM sucrose, 10 mM EDTA, 50 mM imidazole, pH 7.3) in a microtube. The microtube containing the buffer and gill were then frozen at -80^{o}C and maintained at this temperature until the samples could be analyzed. The samples were analyzed according to McCormick *et al.* (2008).

4.1.6 Transfer and grow-out conditions

The study 2 (S0) population (n= 4299) was transferred to a marine cage at a commercial production site using large fish holding tanks on a transport truck, following standard industry practices. The temperature and dissolved oxygen were monitored every 2-4 hours, and dissolved oxygen was adjusted by the fish health technician to maintain standard industry parameters during the 14-hour transfer to the marine cage site.

The fish were reared on a commercial farm site using standard industry practices and maintained in a 70 m polar circle cage (70 m circumference, 15 m depth) for the remainder of the study. The temperature, salinity and dissolved oxygen were recorded at the site on a daily basis.

4.1.7 Statistical analysis

All statistical analyses were performed using STATA (Version 11) software (College Station, TX). The significance level was set at $p \leq 0.05$.

4.1.7.1 Osmolality and gill Adenosinetriphosphatase enzyme activity (ATPase)

Osmolality and ATPase results, obtained at the time of transfer, were compared between the two treatment groups (exposure and control) and the pre-treatment samples using the non-parametric, Kruskal-Wallis test. The data did not have a normal distribution and did not support transformation, therefore, non-parametric methods were required. Significant effects were presented as medians and their confidence intervals. Initial weight and sex were evenly distributed between the two treatment groups due to the random allocation, and could therefore not be confounders for the exposure effect. Pair-wise comparisons between groups were based on the Mann-Whitney test without adjustment for multiple comparisons.

4.1.7.2 Weight difference

The weight difference between the control and exposure groups in the Study 2 (S0) population was compared using a linear regression model. The variable, hatchery precocious parr (male sexual maturity in the hatchery) was included in the model and analyzed for statistical significance, and its two-way interaction was assessed. Sex could not be investigated as a confounder as the fish were not lethally sampled and ante-mortem sex determination was unreliable. Homoscedasticity, normal distribution, linearity and independence were evaluated to ensure the key assumptions of the model were met.

4.1.7.3 Survival analysis

In the Study 2 (S0) population, a Cox proportional hazards model was used to assess the effects of SuperSmolt^{TM} exposure on Atlantic salmon survival for 262 days after the onset of exposure. The assumption of proportional hazards was evaluated using scaled Schoenfeld residuals (Dohoo *et al.* 2009). Initial weight, hatchery precocious parr (i.e., precocious males vs non-precocious males) and treatment group (control or exposure) were included in the model and two-way interactions were assessed for significance.

4.2 Results

4.2.1 Health surveillance

Only opportunistic bacteria were detected from the tissue samples collected at the onset of the study, at the end of the study and from all mortalities. Veterinary diagnostic interpretation did not yield any bacterial, parasitic or viral pathogens of consequence.

4.2.2 Osmolality analysis

The Study 1 (S1) population showed a significantly (P-value = 0.0005) higher osmolality (333.0 Osm/L) in the exposure group than the pre-exposed (323.0) and control (322.0) groups, which in turn were not significantly different from each other (P-value = 0.87) (Table 3.1).

The Study 2 (S0) population showed a significantly (P-value = 0.0001) higher osmolality (343.0 Osm/L) in the exposure group than the pre-exposed (326.0) and control (327.0) groups, which in turn were not significantly different from each other (P-value = 0.75) (Table 3.1).

4.2.3 Adenosinetriphosphatase enzyme activity (ATPase) in gill samples

The Study 1 (S1) population showed a significantly (P-value = 0.03) lower ATPase (5.00 umol ADP/hr/mgprotein) in the exposure group than the pre-exposed (7.38 umol ADP/hr/mgprotein) and control (5.0 umol ADP/hr/mgprotein) groups, which in turn were not significantly different from each other (P-value = 0.79) (Table 3.1).

The Study 2 (S0) population showed a significantly (P-value = 0.0153) higher ATPase (8.15 umol ADP/hr/mgprotein) in the exposure group than the pre-exposed (5.97 umol ADP/hr/mgprotein) and control (5.92 umol ADP/hr/mgprotein) groups, which in turn were not significantly different from each other (P-value = 0.83) (Table 3.1).

4.2.4 Weight difference

All fish in the study 2 (S0) population gained weight during the trial. The control group gained more weight than the exposure study group (P-value <0.001). The mean weight gain for the control and treatment groups were 179.9 g and 168.7 g, respectively (Table 3.2). Due to the short duration of the study 1 (S1) population, the weight difference could not be adequately assessed.

4.2.5 Survival analysis

Of the 4,299 S0 (study 1), Atlantic salmon, 434 mortalities (211 control and 235 exposure) occurred. There was no difference in survival between the control and exposure treatment groups (P-value = 0.392) while hatchery precious parr was a non-significant predictor of survival

(P-value = 0.666) and initial weight was a significant protective factor (HR = 0.971, P-value <0.001) (Figure 3.1). For each gram decrease in initial weight, the hazard of dying increased by a factor of 1.03. Initial weight did have significant non-proportional hazards and, therefore, this was further explored. The non-proportional hazards were non-linear in time but could be modeled by categorizing time into four intervals, by which initial weight had the strongest impact on mortality between 70 and 140 days post-exposure. Time-dependent modeling, of initial weight had virtually no impact on the comparison between exposure and control groups.

4.3 Discussion

4.3.1 Adenosinetriphosphatase enzyme activity (ATPase) in gill samples

ATPase samples are used to determine the Na+ K+ ATPase activity in the gill. This was used as an indicator of the smoltification process. The groups analyzed were the pre-exposed (i.e. before SuperSmolt^{TM}), exposure study group (i.e. exposed to SuperSmolt^{TM}) and the control study group (i.e. no exposure to SuperSmolt^{TM}). The pre-exposed sample was obtained prior to SuperSmolt^{TM}, and the exposure and control treatment group samples were obtained after SuperSmolt^{TM} treatment occurred at the hatchery.

In a saltwater environment, ATPase activity must be stimulated and, therefore, normal ATPase values are higher in saltwater than in freshwater (Wedemeyer, 1996; Strand *et al.,* 2007; Tipsmark and Madsen, 2009). According to Tipsmark and Madsen (2009), freshwater ATPase values are 2.10 +/- 0.12 while saltwater ATPase are 4.24 +/- 0.45. The results for the study 1 (S1) population indicate that the control fish (which did not receive any treatment) had a lower ATPase when compared to the pre-exposed or exposure study groups. However, the ATPase

value was still higher than the saltwater ATPase published values. The fact that ATPase values were not different in the pre-exposed and exposure groups suggests that the exposure study group maintained a physiological status that is associated with smoltification, while the control group may have begun the process of desmoltification (Björnsson *et al.*, 2011).

In the study 2 (S0) population, the ATPase values were higher in the exposed group when compared to both the pre-exposed and control study groups. This would suggest that their physiology was affected by exposure to the SuperSmolt™ process when compared to the control study group. This alteration in ATPase would indicate that the salmon are ready for seawater transfer (Smith, 1993). The ATPase for all groups was consistent with seawater transfer, and the use of this product may not have been required at all. The overall effect of SuperSmolt™ may be due to the change in the external environment. If salts are added to the water prior to transfer, this alone may alter the physiological parameters such as ATPase. Therefore, future studies that make the following comparisons are warranted: SuperSmolt™ treatment versus just adding salts to the water, and salt addition to the water versus a control group.

4.3.2 Osmolality analysis

Osmolality is a measure of the number of dissolved particles per unit of water in plasma. Atlantic salmon in freshwater will have a lower osmolality when compared to those in the marine environment. If consistent, this would be used as an indicator that the fish in the study had undergone the necessary changes for smoltification. The groups analyzed were the pre-exposed (i.e. before SuperSmolt™), exposure study group (i.e. exposed to SuperSmolt™) and

the control study group (i.e. no exposure to SuperSmolt™). The pre-exposed sample was obtained prior to SuperSmolt™ and the exposure and control treatment group samples were obtained after SuperSmolt™ treatment occurred at the hatchery.

Although there was a statistically significant difference between the osmolality values obtained for the study 1 (S1) and study 2 (S0) populations, the values were all within the range expected prior to saltwater transfer. The normal osmolality values for Atlantic salmon in saltwater are 300-350 Osm/L (Saunders *et al.,* 1994) and these were within this range. The differences may be attributed to natural differences in pre-smolt populations. The osmolality values supported the transfer of the smolt populations (exposed and control) to the marine environment. The lack of effect on survival overtime also supports this conclusion. A trial that compares SuperSmolt™ treatment, salt treatment and a control group would also be useful in addressing smoltification.

4.3.3 Weight difference

In the study 2 (S0) population, the control fish gained more weight than the exposed group. This may be due to the specialized diet on which the exposed study group was maintained during the SuperSmolt™ treatment at the hatchery. The entire population was maintained in one marine cage for the duration of the trial, thus providing equivalent environmental conditions and access to feed. However, different feeding rates between the two groups would not have been detected while in the marine cage. The final assessment was performed at day 228 smolt transfer, but it was not a lethal sampling event. Therefore, weight differences between males and females could not be fully assessed.

4.3.4 Survival analysis

In the study 2 (S0) population, there was no difference in survival between the control and exposure study groups. Initial weight at the time of transfer was a significant predictor of survival. This finding is consistent with Handeland and Stefansson (2001), who identified that larger fish appear to develop hypo-osmoregulatory capacity earlier than smaller ones and who also identified higher risk of mortality post-transfer associated with low initial weight. Although lower initial weight is often a predictor of poor survival, it is thought that the physiologic demands of the larger fish in osmoregulatory distress will often cause those fish to die first. The interaction between survival and SuperSmolt^{TM} exposure status was not significant, and this study did not detect any mitigation of potentially poor osmoregulatory function that may lead to eventual mortality by using this physiological modification. However, the significant improvement of osmolality and ATPase may have been due to other interaction factors present in this population. Thus, the opportunity to see the full effect of the altered osmoregulatory status on survival may not have been present in this assessment.

4.3.5 Clinical trials for physiological modifications

The Canadian Food and Drug Act (Health Canada, 2012) states that a drug is any substance or mixture of substances that are manufactured, sold or represented for use for the purpose of one of the following three scenarios.

- the diagnosis, treatment, mitigation or prevention of a disease, disorder, abnormal physical state or the symptoms thereof in man or an animal.
- restoring, correcting or modifying organic function in a man or animal.
- disinfection in premises in which food is manufactured, prepared or kept

SuperSmolt™ is not currently classified as a drug. Based on the definitions and the results of this clinical trial, SuperSmolt™ should be considered a drug, because it modifies organic function and alters the physiological state of the animal. Rigorous clinical trials are required by any company that would like to manufacture or sell a product marketed as a drug to ensure the safety of the product for humans and animals. In the authors' opinion, SuperSmolt™ should be required to have clinical field trials to understand its full impact on health and productivity of fish. Tissue residues produced by a product that contains the same elements routinely found in fish diets or found in their environments does not generate a concern for food safety. However, if there are claims regarding health benefits generated for fish, then the responsibility to substantiate those claims in independent, third-party, peer-reviewed assessments should be mandatory.

4.3.6 Challenges with trial design

This clinical trial faced two significant challenges that could not be overcome. The first was the ability to blind the workers at the hatchery as to which tank received the treatment and which did not. This difficulty was due to the fact that there were different diets for each tank — only one tank received calcium chloride — and so that conductivity reading would be different between the tanks.

The second challenge was the inability to put all the fish (both exposure and control study groups) in one tank in the hatchery due to the water treatment required. This may have led to some tank effect in the hatchery and may explain some of the weight difference observed between the two groups.

Although not an issue in this study, a final challenge that is worth noting for trials of this nature is the general inability to have different water treatments within the same hatchery. This hatchery was a flow-through facility, and so the treatment could be applied at a tank level. A flow-through hatchery is one in which the water comes into the hatchery from a source (well water — as was the case here, or surface water — river or lake) and then flows to the tanks and leaves the hatchery through an effluent system. A recirculation hatchery is one where the water will enter the hatchery from similar sources and flow to the tanks but, instead of leaving via the effluent system, 90% or more of the water is recirculated back to the tanks again. The recirculation hatchery is a challenge in a clinical trial such as this, where water within the tanks needs to be treated differently by the addition of a product to the tank.

4.4 Concluding remarks

Smoltification in Atlantic salmon is a complex process, involving physiological, morphological, biochemical and behavioural changes. SuperSmolt™ claims to change the physiology of Atlantic salmon smoltification. This study showed that SuperSmolt™ alters physiological parameters (ATPase and osmolality) associated with smoltification. In fact, the ATPase and osmolality values measured in the fish from any groups pre-transfer were already suitable for the marine environment, thus bringing into question the usefulness of the product. Further claims of increasing survival and growth after smolt transfer were not substantiated in this independent clinical trial. This study was performed on two different study populations transferred at two industry-standard transfer times. These results do not support the use of SuperSmolt™, as the productivity advantages do not justify the associated costs of royalties, feed and product. More independent, large-scale clinical trials are required to better understand SuperSmolt™ effects in

different aquaculture settings, different species or strains, alternate transfer times, different marine environments and in fish with a different health status than those observed in this study. Studies that compare SuperSmolt™ treatment versus salt alone versus a control group would be extremely important to consider. The physiological parameters measured here (ATPase and osmolality) were chosen because they were the industry standard at the time. Future studies that include other parameters associated with smoltification are recommended (growth hormone, physical characteristics, etc.). The author is unaware of any independent assessments that support the health or productivity claims for SuperSmolt™. Although the results from this study do not support the absence of benefit, neither does there appear to be any peer-reviewed support for the existence of such benefit.

The fact that SuperSmolt™ does change physiological parameters of the fish provides evidence that this product should be evaluated as a drug. This change would require that the company submit additional information that could then be used to evaluate the effectiveness of the product and establish a withdrawal period for this product. This chapter used information from a trial that can now be used by veterinarians and subsequently their clients to make informed decision regarding the use of this product. Furthermore, this study has provided evidence that SuperSmolt™ should be considered a drug and treated as such under the Veterinary Drug Directorate of Health Canada.

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Table 4.1: Study 1 and 2 — Osmolality and ATPase results (medians with 95% Confidence Intervals (CI)) for S1 and S0 populations (study 1 n = 4,525; study 2 n = 4,299)

		Pre-exposed		Control		Exposure	
Variable	SP[1]	M[2]	CI[3]	M[2]	CI[3]	M[2]	CI[3]
ATPase	S1	7.95	6.84 – 8.73	5.00	4.40 – 6.12	7.38	6.34 – 8.69
ATPase	S0	5.97	4.89 – 6.82	5.92	5.08 – 6.62	8.15	7.08 – 8.78
(umolADP/hr/mgprotein)							
Osmolality	S1	323.0	321.4 – 326.6	322.0	319.4 – 327.6	333.0	325.6 – 336.0
Osmolality	S0	326.0	321.4 – 332.0	327.0	322.0 – 332.0	343.0	339.0 – 345.7
(Osm/L)							

[1] Study Population

[2] Median

[3] Confidence Interval for median

Table 4.2: Study 2 — Weight components of S0 dataset (study 1 n = 4,525; study 2 n = 4,299)

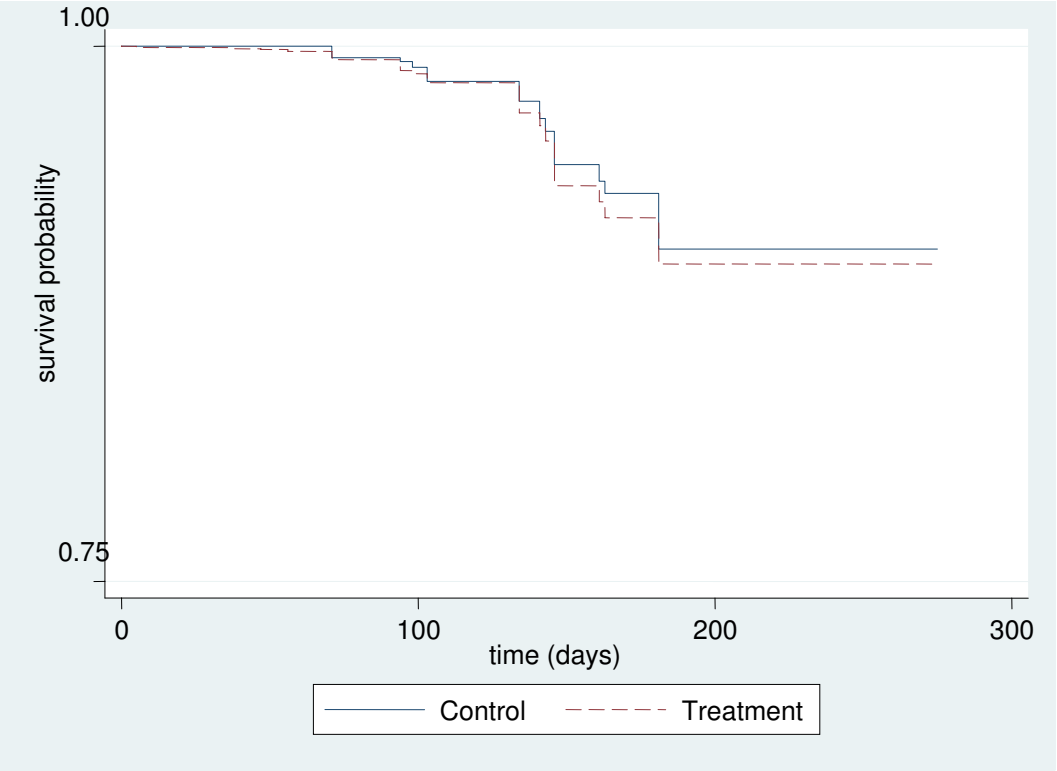
Variable	Group	Mean	St. Dev. [1]	Min	Max	CI[2]
Initial weight	control	42.45	6.70	19	70	42.17 – 42.73
Final weight	control	223.30	52.64	50	420	221.07 – 225.53
Weight diff[3]	control	179.89	50.15	26	363	176.78 – 183.00
Initial weight	exposure	42.61	6.56	21	65	42.34 – 42.90
Final weight	exposure	212.31	48.81	60	430	210.25 – 214.37
Weight diff[3]	exposure	168.67	47.13	17	384	165.76 – 171.56

[1] Standard Deviation

[2] 95% Confidence Interval for mean

[3] Weight Difference

Figure 4.1: Kaplan Meier survival curve for Study 2 (S0) Population for 275 days following allocation to treatment in the hatchery



Chapter 5 Randomized field trial to evaluate fenbendazole treatment efficacy, growth and survival of Atlantic salmon (*Salmo salar*) naturally infected with *Eubothrium crassum* at a marine cage site.

5.0 Introduction

Tapeworms of the genus *Eubothrium* (Nybelin, 1922) comprise eight species, of which the widely distributed *Eubothrium crassum* and *Eubothrium salvelini* affect several salmonid species (Kennedy, 1978). *Eubothrium salvelini* is a freshwater parasite while *Eubothrium crassum* can be found in both freshwater and marine environments. According to Kennedy (1978), *Eubothrium crassum* is subdivided into three races. The European freshwater race completes its lifecycle in freshwater and can be found in migrating salmonids (*Salmo trutta, Salmo salar* and *Onchorhynchus mykiss*). The two marine races (Pacific marine and Atlantic marine) are found in coastal marine environments and were thought to be distinguished only by ecological type. However, Bristow and Berland (1989) concluded that the freshwater and marine races differed genetically into two distinguishable races of *Eubothrium crassum*.

The lifecycle of *Eubothrium crassum* is not fully understood, particularly in the aquaculture environment. The lifecycle, first described in the early 1900s, requires two intermediate hosts. The *Cyclops* sp. (a planktonic species of copepod) was described as the first intermediate host and the perch as the second intermediate host (Kennedy, 1978). Vik (1963) indicated that stickleback (G. *aculteatus*) may also serve as an intermediate host. Experimentally, Saksvik *et al*. (2001b) demonstrated that *Salmo salar* can be infected with marine *Eubothrium crassum* through a marine copepod (intermediate host), requiring 11 months to complete the parasite's lifecycle. The identification of *Eubothrium* species is based on the shape of the scolex and apical

disc, size of the cirrus sac, shape and location of the vitellaria, size of the eggs and the number of testes (Hanzelova *et al*., 2002; Hanzelova *et al*., 2005; Kutcha *et al.,* 2006).

Several factors may affect potential exposure to infective larvae through ingestion of the intermediate copepod host or through ingestion of other infected fish (Hernandez and Muzzal, 1998). Seasonality appears to affect the availability of infective larvae of *Eubothrium crassum*. The infection often occurs in the late summer or early fall with the parasite maturing in the winter and spring, thus releasing eggs to re-infect hosts again in the summer (Hernandez and Muzzal, 1998). Kennedy (1996) described how new generations of *Eubothrium crassum* infected brown trout in a British lake primarily in the spring and summer. The prevalence of infection was related to the size of the parasite and the prevalence and abundance of *E. crassum* at that time of year. *Eubothrium crassum* can complete its lifecycle in either the freshwater or marine environment (Kutcha *et al*., 2006).

The adult cestode resides mainly within the pyloric cecea and proximal intestine of fish possibly leading to a decreased feed conversion ratio, increasing the cost of production, extending the grow-out cycle and impacting the overall health of the animal. Chronic infection with *Eubothrium crassum* on aquaculture farms may result in a 10-20% reduction of growth in Atlantic salmon (Mitchell, 1993). Bristow and Berland (1991) reported that infection with *Eubothrium* sp. resulted in a 10% reduction in growth of infected groups of farmed salmon. A hatchery infected with *Eubothrium crassum* compared the worm burden mass to the condition factor of infected fish and found that small fish were severely impacted by even a low worm burden (Sundnes, 2003). A more recent study showed that parasite-induced changes to the

intestine actually increase food consumption; this increase may be due to upregulation of neuromodulators (Bosi *et al.,* 2005). Increased food consumption, reduced growth and increased production time could induce considerable economic losses for the aquaculture industry. Therefore, safe and effective treatment strategies are sought to control *Eubothrium crassum*.

Fenbendazole and praziquantel have been used as in-feed antiparasiticides by aquaculture veterinarians to treat this parasite. Effective pest management programs depend on the rotation of drugs, which have different modes of action, to prevent resistance from developing. Fenbendazole is a broad-spectrum anthelmintic from the benzimidazole family (benzimidazole methylcarbamates). Fenbendazole produces a degeneration of the parasite microtubule and blocks glucose uptake (Papich, 2007). Praziquantel is a synthetic isoquinolinepyrazine derivative that is highly efficacious against a variety of cestode and trematode parasites. Praziquantel induces a sustained paralytic muscle contraction of the parasite and tegumental disruption (Riviere and Papich, 2009). Although used extra-label (not registered for this specific use), fenbendazole is the only practical treatment available for farmed fish in Canada. The fenbendazole doses most commonly used are either $8mg \cdot kg^{-1}$ once or $5mg \cdot kg^{-1}$ on days 1 and 4 (Personal communication).

Controlled field trials are lacking for assessment of the overall impact of this drug on growth or survival of farmed Atlantic salmon. The objectives of this study were to compare efficacy, growth and survival in farmed Atlantic salmon naturally infected with *Eubothrium crassum* and treated with fenbendazole in a randomized field trial.

5.1 Materials and methods

5.1.1 Study population

A population of pre-market Atlantic salmon, maintained at a marine cage site in Newfoundland and Labrador were found to be naturally infected with the intestinal parasite, *Eubothrium crassum*. This population of fish had been part of a previous study and, therefore, had PIT (Passive Integrated Transponder) tags placed into the intraperitoneal space about 23 months prior to detection of *Eubothrium crassum* infection. The PIT tag, an internal microchip 12 – 14 mm in length, 2 mm in diameter and weighing 0.1 g, is composed of a biocompatible glass encapsulated antenna copper coil and remains dormant until the reader activates it by an electromagnetic field. The microchip generates an alphanumeric code unique to each individual fish.

A total of 1,764 fish were systematic randomly allocated to two cages with 886 fish in the control cage and 878 in the treatment cage. The control group was comprised of 380 males (72 mature) and 423 females (10 mature), while the treatment group was comprised of 360 males (80 mature) and 377 females (3 mature). The sex could not be determined for 17 fish due to poor condition at the time of necropsy.

5.1.2 Sampling

At day 0 (day of randomization), simple random selection was used to obtain a sample of 32 fish from the cage population. The fish were analyzed for underlying health conditions. Mortality collection dives occurred on days 30, 40, 69 and 74, and the fish were harvested on day 78 (Figure 5.1). At the time of the mortality dive, PIT tag number, weight, length, sex and parasite

burden were recorded for each mortality. At the time of harvest, all PIT tags were retrieved and the associated weight, length, sex, parasite burden and other physical characteristics were recorded. Parasite burden was recorded as presence or absence of the parasite. At harvest, a subset of the population (n=150) was selected by simple random sampling, using the PIT tag number, to obtain samples for histology, bacteriology and virology.

5.1.3 Treatment administration procedure

At day 0, the fish were removed from the source cage and then anesthetized with Tricaine Methane Sulfonate (TMS, Syndel International, Vancouver, Canada) to obtain records of weight, length and maturation status (based on milt or egg expression). Each fish was randomly (systematic) allocated to one of two study cages. No other fish were in these cages. After a recovery period of 7 days, the medicated feed containing fenbendazole (SAFE GAURD® [Intervet] — 5 mg/Kg given twice three days apart) was administered to the treatment cage. On day 27 (5 days after the last treatment day), the control fish were reunited with the treated cage by swimming them through a channel between the two cages.

5.1.4 Marine grow-out conditions

The fish were reared on a commercial farm site using standard industry practices and maintained in a 70 m polar circle cage (70 m circumference, 15 m depth) for the remainder of the study. The temperature, salinity and dissolved oxygen were recorded at the site on a daily basis.

5.1.5 Health surveillance

The fish were necropsied whenever presented as a lethal sample, mortality or at harvest. Lethal and mortality samples and a subgroup at the time of harvest were further evaluated for salmonid pathogens. Bacterial cultures were performed using a kidney swab on BA (Blood Agar with 2% salt), TSA (Trypticase Soy Agar) and SKDM (Selective Kidney Disease Media). The BA plates, incubated at 15°C and 22°C, were checked daily for the first week and then held for four weeks. The SKDM plates, incubated at 22°C, were checked daily and then held for eight weeks. TSA plates incubated at 15°C were checked daily and then held for four weeks. Virus isolation was performed using CHSE (Chinook Salmon Embryo) and SHK (Salmon Head Kidney) cell lines. The tissues obtained for virology evaluation were kidney, heart, spleen and gill for either individual fish at the time of harvest or a five-fish pool during all other sampling events. Kidney, heart, spleen, pyloric cecae, gill and liver samples were preserved in 10% buffered formalin and then stained with H & E (Hematoxylin and Eosin) stain for histological examination.

5.1.6 Fenbendazole residue analysis

A subset of the population was obtained at the time of harvest and evaluated for fenbendazole tissue residues. Samples of skin and fillet (100 g) were obtained from 30 fish using simple random selection and maintained at -20°C until they could be analyzed at an external laboratory consistent with the Canadian Food Inspection Agency standards. The method used to analyze the data was based on United States Department of Agriculture (USDA) Method standards (USDA Method BNZ July 1991).

5.1.7 Statistical analysis

All statistical analyses were performed using STATA (Version 10) software (College Station, TX).

5.1.7.1 Parasite analysis

Logistic regression was used to analyze the parasite status data. There is little residual effect of treatment, and the parasite status of the fish would be determined within the first few weeks after treatment. Re-infection by the parasite was not considered possible due to the time of year and the relatively short time span between treatment and harvest. Therefore, parasite status at the time of harvest (or mortality) was considered representative for the post-treatment study period and was used as the outcome variable in the logistic regression analysis. The predictors considered in the model were sex, maturation status, initial weight and treatment group. As the fish were randomized late in the fall, maturation status was considered stable over the course of the study. Initial weight was obtained at the time of the randomization and any effect of maturation status on initial weight would have already occurred, making initial weight an intermediate (intervening) variable for the effect of maturation (Dohoo *et al*., 2009). Therefore, initial weight was centered within each maturation group so that the full effect of initial weight and maturation status could be evaluated. All the variables included in the model were analyzed for statistical significance and all two-way interactions were considered. The model was evaluated for fit using the Hosmer-Lemeshow Goodness of Fit test.

5.1.7.2 Survival analysis

Due to the few time points at which the mortality dives produced mortalities, the analysis was carried out as a discrete time survival analysis with three time periods: from day 27 to day 40,

from day 40 to day 69, and from day 69 to day 74. Three logistic regression analyses using mortality as the outcome variable were analyzed — one for each time period — including all fish alive at the beginning of the period (Dohoo *et al.*, 2009). Initial weight, maturation, sex and treatment group and their interactions were analyzed in each period. Parasite status was determined to be an intermediate variable for the association between treatment and mortality and was omitted from the analysis of the treatment effect. Additional analyses were performed by adding parasite to the model to assess the direct effect of parasite status on mortality. The final models were evaluated as described previously for parasite analysis.

5.1.7.3 Weight difference

At the time of harvest, weight differences from study onset were compared between treatment groups using the non-parametric Mann-Whitney test. Significant effects were represented by medians and their confidence intervals.

5.2 Results

5.2.1 Health surveillance

The health samples (those collected at the onset of the study, at harvest and approximately 20% of the mortalities that occurred) did not reveal any significant findings consistent with bacterial or viral pathogens or disease. Prior to the study, the population was confirmed to have infection with *Eubothrium crassum* in one or more of the following organs: stomach, pyloric cecae and intestine. Prior to treatment, the prevalence of *Eubothrium crassum* was 25% (CI: 11.5 – 43.4). The fish were confirmed to have concomitant infestation with sea lice (*Lepeophthrius salmonis*) prior to harvest, resulting in skin lesions, particularly in the head region. The skin lesions may

have resulted in dysregulation, and osmoregulatory function was impaired in this group of fish. There was no evidence of sea lice present at the time of randomization to fenbendazole treatment. Furthermore, *Lepeophthrius salmonis* are not known to be aggressive feeders at low water temperatures (less than 5°C). Although winter weather constraints caused some delays, early harvest was chosen as the most appropriate mitigation strategy for the study fish as soon as elevated mortalities were noted.

5.2.2 Parasite analysis

The odds ratio (OR) for the treated group was 1/7.29, indicating a fish in the treated group to be 7.29 times less likely to have the parasite when compared with the control group (Table 5.1). The prevalence of fish with parasites in the treated group was 6.5% when compared with the control group, which had a prevalence of 32%. Maturation also affected the risk of having parasites: immature fish were 2.95 times more likely to have parasites than mature fish (Table 5.1). The initial weight was a significant risk factor (OR = 0.79 per incremental kg) on parasite burden (Table 5.1), with larger fish being less likely to be infected than relatively smaller fish. All two-way interactions were considered but determined to be non-significant.

5.2.3 Appetite observations

Based on farm feeding records, the group given in-feed fenbendazole were observed being off-feed (less than 50% of the control group) for a period of one week when compared with the control group.

5.2.4 Survival analysis

Analysis was performed on 1,557 fish in total, as portions of the mortalities were too old to obtain an accurate weight, length or to determine the sex of the fish. The first time period for survival analysis was defined as starting immediately after the fish were combined together in the same cage (day 27) and ending at day 40. The initial weight, maturation status and treatment group were all significant predictors of mortality during this time period (Table 5.2). A mature fish was 15.7 times more likely to die during this time period when compared to a non-mature fish if all other variables were equal. The treatment group (OR = 3.32) and small initial weight (OR = 0.52 per incremental kg) were also significant risk factors.

The second time period (day 40 to day 69) had the same significant risk factors, but all estimates were closer to the null (Table 5.2). By day 69, there were very few mature fish left in the population. The total number of mature fish in the population was 151, which decreased to 113 by the next time period. Only 14 survived through to harvest. This resulted in a reduced ability to detect associations related to maturation status later in the study. The only significant effect during the final time period was for treatment group (OR = 2.55). For all time periods, if parasite status is added to the model, then the estimates and their magnitude of significance do not change substantially.

5.2.5 Weight difference

Approximately 75% of the harvested fish lost weight during the trial, with the treated group losing more weight than the control group (P-value = 0.0001). The median weight loss for the

control and treatment groups were 0.12 kg (CI: 0.098-0.155) and 0.35 kg (CI: 0.325 – 0.395), respectively.

5.2.6 Fenbendazole residues

The high-performance liquid chromatography (HPLC) results revealed that all 30 samples obtained at harvest were below the detection limit of 0.001 ppm.

5.3 Discussion

5.3.1 Mortality

The mortality occurring in the study population was likely due, at least in part, to skin damage caused by sea lice, *Lepeophthrius salmonis*, leading to osmoregulatory stress. Sea lice infestations in populations of farmed fish are due to natural infection from wild salmonids. Once farmed fish become infected, the sea lice will reproduce effectively within the cage site due to the close proximity of many suitable hosts. The common practice on aquaculture farms is to treat sea lice once they reach a threshold during certain times of the year when infection is anticipated or when protection of wild stocks warrants it (Brooks, 2009). In this instance, sea lice infestation in the study population was most likely sourced from wild salmonids since there was no evidence of sea lice at the initiation of the study and water temperatures and timing were not conducive to development of mobile stages from attached chalimus stages of *Lepeophthrius salmonis*, which caused the observed skin damage. There are several different mitigation strategies in place for treating sea lice, including early harvest, towing cages into brackish or freshwater and bath or in-feed treatments (Lees *et al*., 2008). The only treatment registered in

Canada at the time for sea lice was emamectin benzoate (SLICE®), which required a 68-day withdrawal period. Early harvest was the chosen course of action.

5.3.2 Treatment effects

As demonstrated by the decreased prevalence of parasites, in-feed fenbendazole was effective in treating *Eubothrium crassum*. However, the fish were observed to go off-feed for a period of one week compared to no change in feeding behaviour for the control group. The described inappetence was noted once all the medicated feed had been fed to the fish in the treated group. This altered appetite of salmonids treated with fenbendazole has been anecdotally observed in the aquaculture industry on several occasions and the period can vary from one to six weeks.

Treatment was associated with an increased risk of mortality compared to the control group, an association that was consistent over the study period. This elevated mortality of the treated fish may be related to several factors, including *Eubothrium crassum* death resulting in an immune response after treatment. The immune response post-treatment may have resulted in a cytokine storm or another immune related consequence. In higher vertebrates it has been documented that immune evasion mechanisms can cause pathogenic consequences in the host and this can lead to death (Schmid-Hempel, 2008). The actual death of the worms may also be responsible for the increased mortality. In dogs and cats treated for heartworm (*Dirofilaria immitis*) the simultaneous death of adult worms can cause acute disease associated with an inflammatory response or thromboembolic events (González-Miguel *et al.*, 2012). It is reasonable to assume that the acute worm death in this case could cause a similar inflammatory

response but within the gut as well as mechanical damage and obstruction may be possible if the worm burden was severe. Concurrent sea lice infection when compromised by recent treatment or external parasites is also possible. Both the control and treated groups were naturally infected at the same time with *Eubothrium crassum* months prior to sea lice infestation or treatment. As both the control and treated groups were maintained in the same cage, the level of sea lice exposure was similar due to the same environment for all study fish. Bristow and Berland (1991) reported that increased attacks in Norway of *Ichthyobodo* spp. on farmed salmon were observed when *Eubothrium* sp. were present. Infection with *Eubothrium* sp. may induce immunosuppression in the host (Boyce and Clarke, 1983). However, in this field trial, cestode prevalence was assumed to be equivalent based on random assignment to the treated and control groups. Therefore, the impact of the *Eubothrium* parasite would be similar in both groups. The increased stress of treatment may have resulted in a multi-factorial response leading to the mortality. It is also possible that the increased mortality was due to a drug reaction but further work and studies would be required to determine if this is a factor or not. The distinguishing factor was the treatment with fenbendazole, which resulted in a differential mortality through an as yet unexplained mechanism.

5.3.3 Other effects

The majority of the sexually mature fish died during the first two time periods, and very few mature fish remained by day 69. The small number of mature fish remaining at day 69 explains the inability to detect a significant effect of maturity during the final time period. Field observations suggest that mature fish may be compromised by their inappetance during seasons (i.e. fall) when they they would normally be entering freshwater to spawn. Their fat stores are

generally decreased over time reflecting a negative energy an nutrient balance. Although difficult to definitively conclude how this might influence their susceptibility to infectious or non-infectious diseases, it is logical that mature fish may experience more impactful consequences from any additional health challenge. The decreased risk of parasite burden in mature fish that was detected in this study may be due to the decreased appetite experienced during gonad development, preventing infection by ingesting the intermediate host. However, we cannot dismiss the possibility that physiological changes in maturing fish may lead to decreased survival of *Eubothrium crassum.* Low initial weight was identified as a risk factor in the study (Table 5.1). This would be consistent with experiences at the marine cage site, where smaller fish often succumb first in the presence of a significant disease challenge, such as sea lice parasitism.

5.3.4 Weight difference

Both the control and treated groups lost weight during the follow-up periods. This was most likely due to sexual maturation status, decreased feeding when water temperatures decrease, and concurrent sea lice infection. The weight loss in the treated group was significantly higher than in the control group; the possible reasons for this increased weight loss are inappetance in the period immediately after treatment and decreased parasitism (decreased parasite mass). The timing of this clinical field trial (i.e., during decreasing water temperatures) and the proximity to harvest weight prevented this study from fully evaluating the effect on growth when the parasite burden was removed. If this study was repeated during warmer water temperatures and the grow-out period post-treatment was longer, a growth differential between the treated and control group may have been observed. Saksvik *et al.* (2001a) found a growth difference of 21.6% between infected and uninfected Atlantic salmon that were

experimentally infected with *Eubothrium crassum*. These findings support the conclusion that a longer-term trial is required to fully evaluate the effect on growth post-treatment.

5.3.5 Fenbendazole residue analysis

As this was the first time this treatment had been used within this region, residue sampling at the time of harvest was required by the prescribing veterinarian. The HPLC data showed that fenbendazole was undetectable at a detection limit of 0.001 ppm at the time of harvest. A depletion study showed that fenbendazole was not detectable after 96 hours in the skin and fillet of rainbow trout and Atlantic salmon, respectively (Isofidou *et al*., 1997). The HPLC results were obtained on day 78. Therefore no fenbendazole residue would be expected in the fillet or the skin.

5.4 Concluding remarks

The lifecycle of *Eubothrium crassum* in the marine environment is still poorly understood, and it is thought that an intermediate copepod is required to complete the lifecycle. In an aquaculture setting, the exposure to infective stages, enhanced by the close proximity of the fish, allows the parasite to infect the host without an intermediate host. This scenario may occur through cannibalism. Exposure might also be decreased due to the preference of the salmon host to pelleted feed over the intermediate copepod. This study concludes that treatment with fenbendazole at 5 $mg \cdot kg^{-1}$ on days 1 and 4 is effective for reducing the prevalence of naturally occurring *Eubothrium crassum* at a marine cage site. However, the results from this study demonstrated a differential mortality in the treated group and a possible interaction between treatment and concurrent disease. Randomly designed field trials provide aquaculture

veterinarians with crucial evidence to support the making of clinical decisions regarding the benefit versus adverse effects of mitigation strategies.

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Figure 5.1: Study events according to time

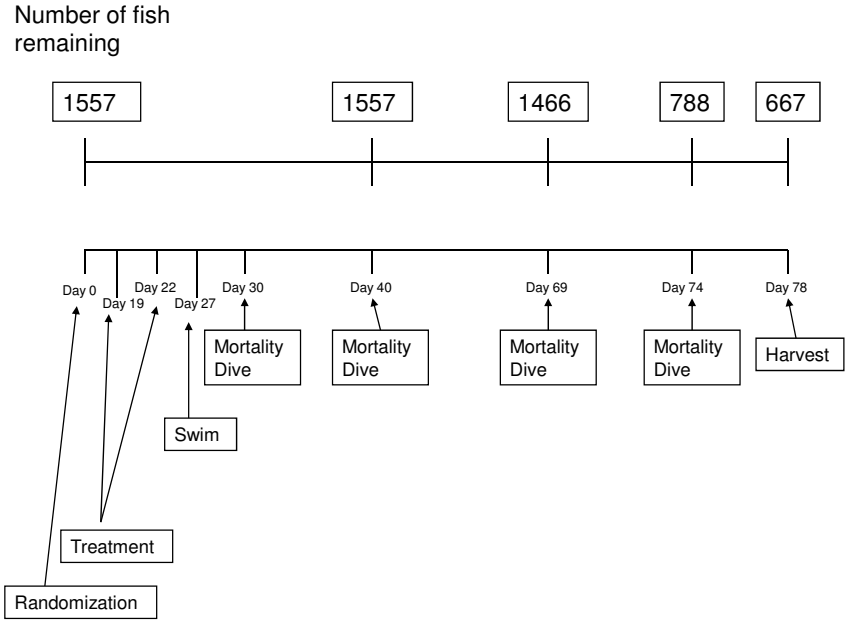

Table 5.1: Parasite burden risk factors

Variable	Odds Ratio	Standard Error	95% CI[1]	P-value
Treatment Group	0.14	0.03	0.10, 0.20	<0.001
Maturation Status	0.34	0.11	0.18, 0.65	0.001
Initial Weight	0.80	0.06	0.68, 0.93	0.003

CI[1] – Confidence Interval

Notes:

1) Maturation status was determined at the time of randomization
2) Initial weight was centered within each maturation group

Table 5.2: Risk factors associated with mortality (discrete time survival analysis)

	Days 27-40			Days 40-69			Days 69-74		
	OR[1]	SE[2]	P[3]	OR	SE	P	OR	SE	P
MS[4]	15.72	4.08	<0.001	9.87	2.84	<0.001			>0.05
TG[5]	3.32	0.90	<0.001	2.64	0.29	<0.001	2.55	0.52	<0.001
IW[6]	0.52	0.72	<0.001			>0.05			>0.05

OR[1] – Odds Ratio

SE[2] – Standard Error

P[3] – P-value

MS[4] – Maturation Status

TG[5] – Treatment Group

IW[6] – Initial Weight

Note: Maturation status was determined at the time of randomization

Chapter 6 Conclusion

6.0 Evidence-based veterinary medicine

Aquaculture has grown rapidly to meet the increasing demands for aquatic products. Aquaculture production creates employment opportunities in rural and coastal communities (Tidwell and Allan, 2001; Bostock *et al.,* 2010; Thompson and Subasinghe, 2010). Employment in rural communities and the need for protein sources for the world are some of the driving forces for continued investment and development of aquaculture. When compared to other food-animal production systems, aquaculture is still viewed as new and innovative. As with all species, the health of the animals must be considered. Veterinarians play a crucial role in the sustainability and development of aquatic species as a food source.

Professionals in many fields of veterinary medicine often employ evidence-based veterinary medicine (EBVM) in an informal way; this is especially true in aquatic veterinary medicine due to its relatively brief history. EBVM is the process of integrating clinical expertise and the best available research evidence (Cohen, 2009). Aquaculture veterinarians play a crucial role in aquaculture production in both established and newly emerging species. Aquaculture veterinarians work with food-producing animals and, therefore, one of their roles is to protect human health and the safety of the food supply by keeping animals healthy. Another role of the aquaculture veterinarian can be regulatory within the federal or provincial/regional government. Within government, they will have many responsibilities that will vary daily. These responsibilities will include, but will not be limited to, providing guidance to policy-makers, assisting in risk analysis, advocating for research, regulating aquaculture activities, conducting surveillance, assisting in export/import or conducting outbreak investigations. The aquaculture

veterinarians employed by the province of Newfoundland and Labrador provide both front-line medicine for the industry as well as play a regulatory/advisory role for the province. Private or company aquaculture veterinarians provide veterinary care to their clients, which includes, but is not limited to surveillance, diagnostic interpretation and mitigation strategies. Aquaculture veterinarians can also play a role in other areas such as research, pharmaceutical development, diagnostic testing, nutrition, genetics and vaccine development.

EBVM is just one of the tools that aquaculture veterinarians will use in practice. This thesis was developed based on opportunities and needs established by the NL industry and their veterinarians. One of the fundamental principles of EBVM requires that clinical activities must be based upon well-designed studies of spontaneous disease. There are many challenges to performing these activities in an aquaculture setting. Challenges in aquaculture research are focused on the logistics of fish farming and prospective data collection. Novel pathogens and newly emerging aquaculture species make it difficult to predict parameters that need to be recorded (possible confounders). New diagnostic techniques for emerging species and their pathogens are challenging, as well as the mitigation required (i.e., treatment and vaccines). To facilitate the practice of EBVM, the aquaculture industry and their practitioners must work together to develop applied research and then disseminate in peer-review publications.

It is essential that well designed, peer-reviewed, randomized controlled trials be published to ensure that quality information is available to those making decisions about clinical interventions. In the practice of veterinary medicine applied to aquatic animal health, the volume of RCT or other sources of information on which to base management decisions is

scarce. This thesis addressed evidence gaps in commercial aquaculture as they were identified. As aquaculture grows throughout the world, challenges requiring evidence generation for management and policy decision improvements will be required. Sound health management will continue to demand scrutiny to sort out good decisions based on evidence from decisions based on assumptions. Evidence-based veterinary medicine will continue to be employed by veterinarians to make sound health management decisions. To ensure that these decisions are based on the best and highest quality evidence, RCT and publication of these results are required. This will require commitment from industry and their veterinarians and, as demonstrated throughout this thesis, such a commitment is possible and mutually beneficial.

6.1 Clinical investigations

Although not formally EBVM, practicing veterinarians will use all the information available to make the best management, mitigation and treatment decisions. Veterinarians will seek out evidence from RCT to help in the decision-making process. Randomized controlled trials provide the foundational evidence on which clinical management decisions are made. Much of this thesis is based on actual trials or generating methods that were required to perform those trials. First, generating an ability to randomize individuals to treatment groups required further evidence that PIT tagging methods would cause no detectable harm in Atlantic cod. Second, during the early 2000s, understanding factors related to nodavirus outbreaks were extremely important in Atlantic cod as this virus was responsible for significant morbidity and mortality in hatcheries. Lastly, two opportunities to assess treatment outcomes in salmonid health management resulted in the analysis for *Eubothrium crassum* treatment and SuperSmolt^{TM} use in the hatchery.

6.2 Evidence to support trial execution: Passive Integrated Transponder (PIT) tagging in Atlantic cod

To minimize animal use in clinical trials and increase statistical power by evaluating fish on an individual fish level, Passive Integrated Transponder (PIT) identification was chosen as part of the RCT in this thesis. The first objective of the study was to determine the placement of these tags in Atlantic cod given their relatively small abdominal space and large liver. The second objective was to determine if there was any difference in growth and survival when PIT tags were used in a population. By demonstrating to the producer that PIT tagging could be performed on Atlantic cod with minimal risk or impact on growth and survival over the short term, the use of this identification technique could be employed in future trials in support of further EBVM in cod.

The results from this study indicated that PIT tag placement located left of midline and 2 mm cranial to the anal pore into the intraperitoneal cavity of 20 g cod had no detectable negative or adverse effects on weight and survival in the short term. This study resulted in 100% tag retention and demonstrated that this tagging technique could be employed in Atlantic cod and may be used as a tool in research, broodstock identification or disease surveillance. Although this placement and the technique used to place the tag showed no detectable difference, there are other possible locations and surgical techniques that can be used. It would be useful to conduct a study comparing the different tag placement locations as well as how the tags can be placed (hypodermic needle, scalpel, etc.) to determine if one method had a better outcome when compared to the others. In addition, different species could be evaluated, such as salmonids or other gadoids (e.g., halibut).

6.3 Evidence from trials

6.3.1 Nodavirus trial

Evidence on decisions regarding vaccination of Atlantic cod is primarily derived from work done in other species. The vaccines available are multi-valent vaccines developed for salmon. This assumption comes from the fact that the immune system of cod is not fully understood and that the innate immunity plays an important role in this species. Cod do not seem to have the same adaptive response as salmonids, with specific antibodies generated in response to a vaccine. The non-specific pathway is activated when cod are vaccinated, and it is thought that a generalized immune response would provide protection for the cod.

When a natural outbreak of nodavirus occurred in a group that had been randomized to salmon vaccines (i.e.,no nodavirus antigens), a project was established to examine the possibility that nodavirus-positive fish would show a difference in survival between previously vaccinated and non-vaccinated control groups of cod. The dip vaccine, containing a bacterial antigen, may be protective in a viral outbreak as the innate immune system can be stimulated by the vaccine (Magnadóttir *et al.,* 2001).

The vaccine showed an indication of being protective and was most evident after approximately 20 days. This delayed response is expected after a vaccine is employed in mammals or fish generating a specific immune response to a pathogen. Atlantic cod do not mount a specific immune response but rather a non-specific response (Whyte, 2007). It is assumed that a delayed response is required in Atlantic cod, but both the length of time required and the duration of

effect remain unclear. The effect of vaccine was not consistent across all tanks, and, given the small number of tanks, it would be advisable to confirm the findings in a study with additional tanks. To better quantify this, individually marked fish would be ideal, but often affected fish are too small for most marking techniques. All vaccines used in aquaculture settings should be critically evaluated for the species in which they will be utilized.

In the future, more studies looking at the immune response of Atlantic cod post-vaccination are warranted. Investigations related to the number of degree days required to provide protection, how long the vaccine is effective for and the age at which the vaccine should be used are important to consider. Other possible considerations are whether booster vaccines are required and the delivery method. Providing a vaccine through different modalities at different life stages may prove to be useful and effective. For example, live feed could be used to deliver a vaccine during the larval rearing stages by “feeding”, or enriching, the rotifers and artemia just prior to feeding. Dip vaccines may be used at the smaller sized fish (5 – 10 g), and intraperitoneal vaccination maybe used at larger fish sizes prior to marine water entry. If vaccination provides a short duration of protection at sea, it is possible to vaccinate in the marine environment either via feed or intraperitoneal injection. Feed delivery would be easy to administer and less stressful on the fish, but it is difficult to control how much vaccine is delivered to the fish during feeding (i.e., aggressive large fish will eat first and more while smaller less aggressive fish may not receive any feed). Intraperitoneal vaccination at sea would provide the fish with a fixed dose of the vaccine, but this is labour-intensive and more stressful on the fish than an in-feed treatment. It is clear from these questions and suggested trials that information obtained that

can feed into EBVM is an important tool that practicing veterinarians must have available to them.

6.3.2 SuperSmolt™ trial

SuperSmolt™ is considered a nutraceutical, currently supplied by Europharma, and its use is promoted in Atlantic salmon aquaculture industry as a means to enhance the osmoregulatory transition of Atlantic salmon smolt as they are transferred to seawater. Some of the claims of SuperSmolt ™ include earlier attainment of standard S0 size, larger S0 fish, synchronized smolt transfer schedule and increased survival with decreased risk of disease after seawater transfer (Europharma, 2012). Should these effects be conferred after SuperSmolt™ treatment, such a nutraceutical would be beneficial. However, there is little independent evidence to support these claims. The NL salmonid industry was using this product on a small scale and poised to use it more widely if the appropriate use could be defined. AN RCT was initiated at a hatchery with the intent to follow fish through seawater transfer, where the effect would be most evident.

The study showed that SuperSmolt™ alters physiological parameters (ATPase and osmolality) associated with smoltification. However, further claims of increasing survival and growth after smolt transfer were not demonstrated in this trial. This study was performed on two different study populations transferred at two industry-standard transfer times. These results do not support the additional costs of using SuperSmolt™ without generating a productivity benefit. This was a relatively small trial and does not conclusively demonstrate its ineffectiveness in all production situations. However, there was no advantage detected in this typical NL salmon farming situation. More independent large-scale RCT are required to better understand

SuperSmolt™ effects in different aquaculture settings, in different species or strains, at alternate transfer times, in different marine environments and in fish with different health statuses. This study highlighted the need to critically evaluate all aspects of salmonid production through the use of RCT. This information will better inform the EBVM process and potentially help the profitability of the producer.

The fact that this study did show a change in the physiological response in the salmon indicates that the classification of SuperSmolt™ as a drug may be warranted. This is due to the fact that SuperSmolt™ modifies organic function in an animal; this meets the definition of a drug set by the Canadian Food and Drug Act (Health Canada, 2012). When a product meets this definition, additional testing and trials are required before it can be used. This critical evaluation is warranted in this case.

6.3.3 Eubothrium crassum trial

Eubothrium crassum, a cestode found in the pyloric cecae and proximal intestine of wild and farmed Atlantic salmon, has a wide distribution in marine and freshwater environments (Kennedy, 1978). Although not quantified, the adult cestode likely leads to a decreased feed conversion ratio, increased cost of production, extended grow-out cycle and can impact the overall health of the animal (Mitchell, 1993). The lifecycle of *Eubothrium crassum* in the marine environment is poorly understood, and it is thought that an intermediate copepod is required to complete the lifecycle. Treatment with a pharmacologic agent known to reduce cestode numbers should improve survival and growth. Further collection of evidence to support its widespread use in aquaculture requires that all effects on growth and survival be evaluated in

218 an RCT. An Atlantic salmon population that was previously PIT tagged was diagnosed with a
219 natural infection of *Eubothrium crassum*. Thus, the opportunity presented itself to evaluate
220 treatment effects by randomizing individual fish to treatment or control (no treatment).

221

222 The study concluded that treatment with fenbendazole at 5 $mg \cdot kg^{-1}$ on days 1 and 4 was
223 effective at reducing the prevalence of naturally occurring *Eubothrium crassum* at a marine cage
224 site in NL. However, the results from this study demonstrated a differential mortality in the
225 treated group and a possible interaction between treatment and concurrent disease. Although
226 this trial confirms that *Eubothrium* prevalence is reduced by fenbendazole treatment, it also
227 confirms the need to examine all evidence related to benefits and possible adverse effects.
228 Concurrent disease interactions are difficult to explore under field conditions, but mortality
229 outcomes should have been similar in both groups based on randomization evenly distributing
230 any immeasurable confounders. The fact that mortality was greater in treated fish suggests that
231 this treatment should not be assumed entirely beneficial under all circumstances. Future use of
232 this product must be considered with this possible negative side effect. This study highlighted
233 the need of RCT and their use in EBVM.

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6.5 References

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